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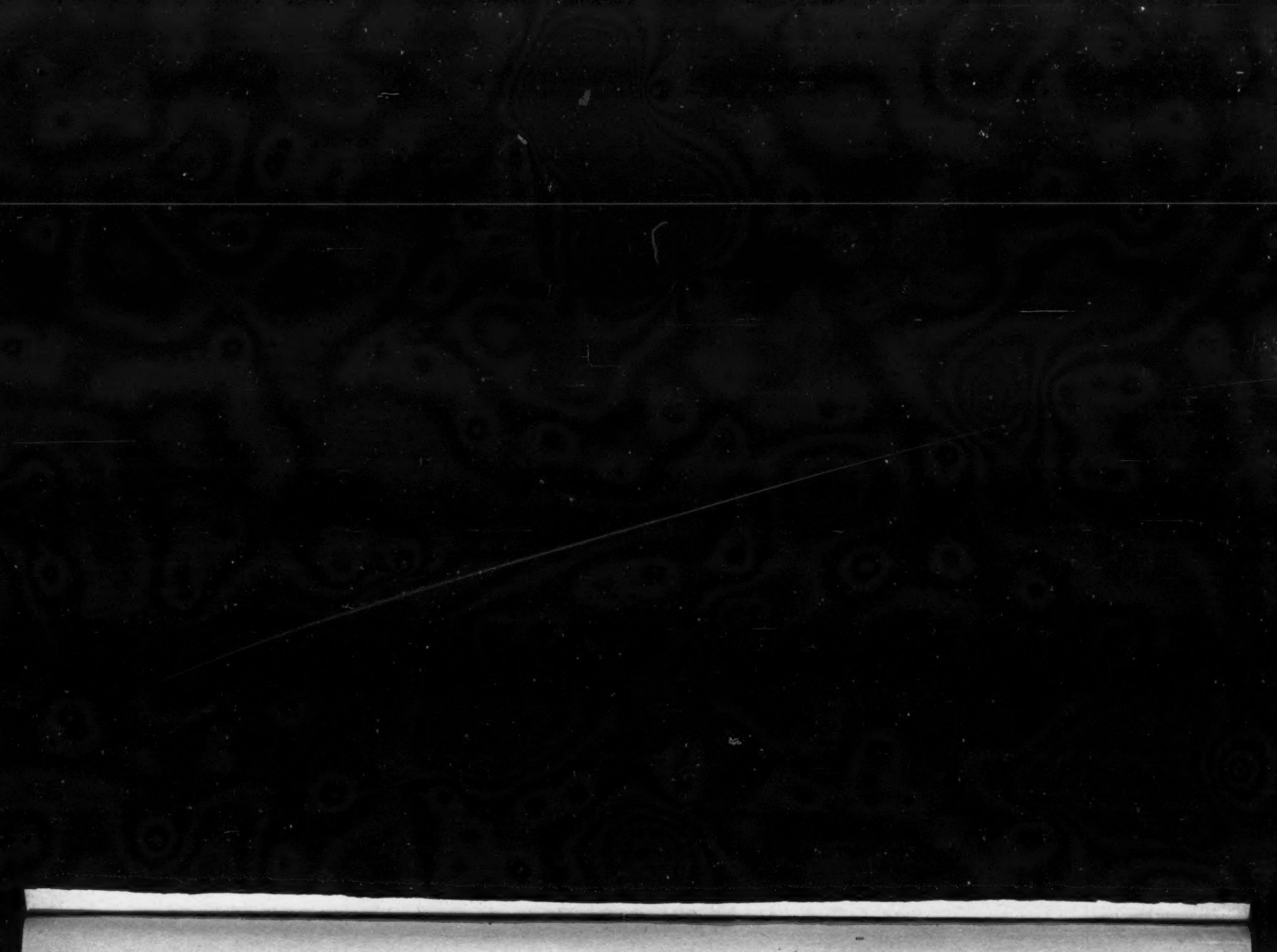

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THE MECHANISM OF ACTION OF ANTICHOLINESTERASE DRUGS

BY

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During the past few years much new work on anticholinesterase substances has been published as a result of the new interest in the subject produced by the discovery of di*iso*propylfluorophosphonate (DFP) by McCombie and Saunders (1946). Many discrepancies of detail between the behaviour of individual cholinesterase inhibitors have been noted and have occasioned serious doubts as to the validity of the hypothesis that the physiological action of these substances is solely a consequence of cholinesterase inhibition. One source of error was greatly clarified by Mendel and his co-workers (Mendel, Mundell, and Rudney, 1943; Mendel and Rudney, 1943 and 1944; Hawkins and Gunter, 1946; Hawkins and Mendel, 1947), who showed that the term "cholinesterase" has been applied in the past to enzymes of two, and possibly more (Augustinsson, 1948), distinct enzymological species—termed by them "true" and "pseudo" cholinesterases, distinguishable by specific substrates. They have shown that these enzyme types differ strikingly in their distribution, their sensitivity to inhibitory agents, and their optimum substrate concentrations, and that the appearance of the pharmacological effects of anticholinesterases is related to inhibition of the "true" cholinesterase. Only when the physiological response measured is that to acetylcholine carried by the blood stream does the pseudocholinesterase in the plasma seem to be important (Heymans, Verbeke, and Votava, 1948) in determining the magnitude of acetylcholine responses. Despite this very important advance many anomalies remain to be explained, and it is the purpose of this paper to discuss the kinetics of cholinesterase inhibition by various agents and the light this sheds on our interpretation of cholinesterase inhibition under strictly physiological conditions. Some aspects of the kinetics of cholinesterase activity and inhibition have been considered by a number of workers (Straus and Goldstein, 1943; Goldstein, 1944; Mazur and Bodansky, 1946; Nachmansohn, Rothenburg, and Feld, 1947; Augustinsson, 1948; Brauer, 1948; Jansen, Nutting, and Balls, 1948; Mackworth and Webb, 1948; Nachmansohn, 1948), but there has been little attempt to interpret these results in terms of prevailing physiological circumstances, and in consequence the design of experiments on cholinesterase activity has usually been inadequate to provide relevant data. In the account that follows attention will be concentrated on the contrast between the modes of action of the two main groups of anticholinesterases, typified by eserine and tetraethyl pyrophosphate, with notes on such differences from these patterns as are found with other inhibitors.

Materials and Methods

The enzyme preparations used were: (1) fresh oxalate human plasma which contains predominantly pseudocholinesterase; (2) washed human red blood cells, lysed by the addition of 4 volumes of 0.025*M* $NaHCO_3$, containing only true cholinesterase; (3) in some experiments a highly purified bovine red cell true cholinesterase, obtained from Dr. M. L. Tainter, has been used, each mg. of which could hydrolyse 3 mg. of *d*(+) acetyl-β-methylcholine chloride per minute. The activity of the enzyme was estimated in the Warburg manometric apparatus with 0.025*M* $NaHCO_3$ as medium equilibrated with 95 per cent N_2 + 5 per cent CO_2 gas mixture at 37° C. The enzyme solution was normally placed in the main compartment of the vessel, and the substrate and inhibitor, each dissolved in 0.2 ml. of bicarbonate solution, were placed in separate side arms. The total volume of fluid used was always 3 ml. The usual final concentration of substrates were 0.025*M* *dl*-acetyl-β-methylcholine chloride, 0.007*M* benzoylcholine chloride, and 0.02*M* acetylcholine chloride. Where other concentrations of substrate have been employed they are mentioned in the text. In all experiments corrections for non-enzymic hydrolysis of the substrate were applied. Dilute solutions of the enzyme inhibitors have been made immediately before use from stock solutions, or with tetraethylpyrophosphate (TEPP) and di*iso*propylfluorophosphonate (DFP) from the pure substances.

Results

There is a tendency in pharmacology to assume an identical mechanism for two drugs that have similar actions if their dose-response relationships are similar. In Fig. 1 data for eserine and TEPP inhibition of true cholinesterase are given. Apart from the curve for TEPP being much steeper than

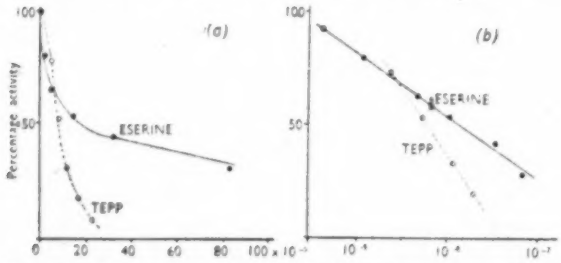


FIG. 1.—Human red cell cholinesterase. Substrate: 0.025*M* acetyl-β-methyl choline chloride. Incubation of inhibitor and enzyme for 20 min. before addition of substrate. Ordinates: velocity of hydrolysis of substrate as percentage of uninhibited control rate. Abscissae: concentration of inhibitor in g./ml. (*a*) Arithmetical scale, (*b*) log. scale.

that for eserine and consequently appearing nearly linear over the range 0–70 per cent inhibition, there is nothing in such data to suggest the striking difference in kinetics of action that will be discussed. Such differences of dose-response slope are very commonly encountered in pharmacology among apparently closely related drugs, notable examples being found in the central analgesic and local anaesthetic series, without arousing any doubts as to a unitary mechanism of action. It should be noted that this steep dose-response curve may account for a feature of TEPP action—namely, that its pharmacologically effective dose is much closer to the lethal dose than is the case with eserine or prostigmine.

Kinetics of Eserine Action

(*a*) *Rate of combination of eserine and enzyme in the absence of substrate*

At present it is not possible to measure this reaction directly; it can only be inferred from indirect data. If eserine is left in contact with the enzyme for 1, 5, or 20 min. at 37° C. before addition of substrate the subsequent rates of hydrolysis are indistinguishable, but such information is not helpful. The rate of combination in the absence of substrate—i.e., under non-competitive conditions—however, must be faster than in the presence of substrate. It will be seen in the next section that under these conditions combination occurs fairly rapidly.

(*b*) *Rate of combination of eserine and enzyme in the presence of substrate*

Fig. 2 shows some typical results illustrating the rapidity with which eserine reacts with cholinesterase when it is actively splitting acetylcholine; it will be seen that an equilibrium rate is reached in about 10 min. after adding eserine. The rate at which equilibrium is reached is dependent, however, on the concentration of inhibitor employed; with lower concentrations the rate is slower, as was predicted on theoretical grounds by Goldstein (1944), but a steady state is finally attained whose position is determined competitively by the concentrations of inhibitor and substrate.

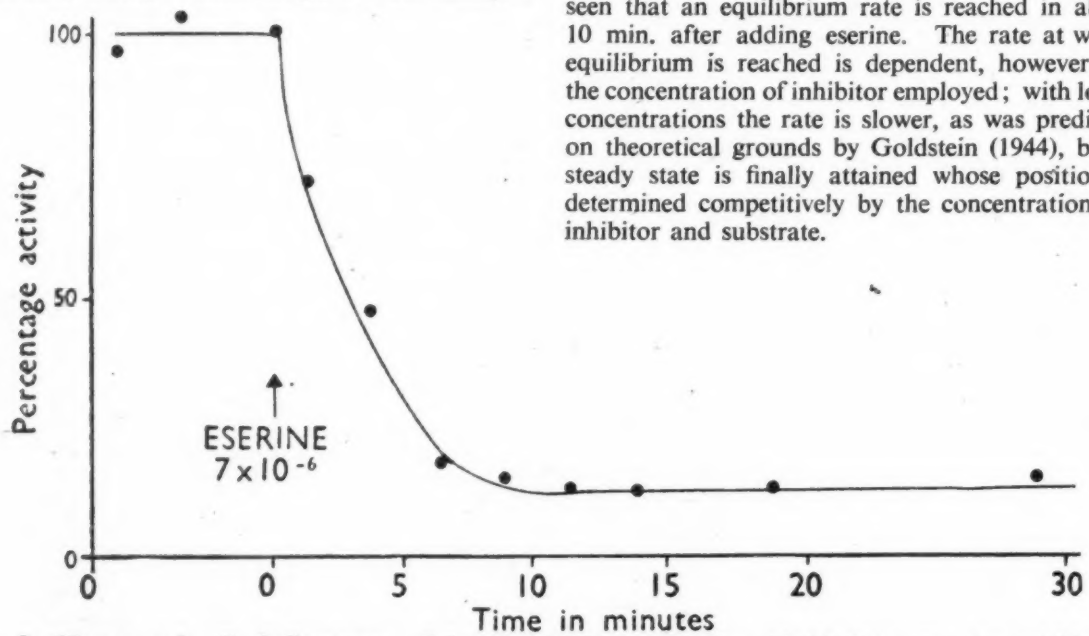


FIG. 2.—Human red cell cholinesterase. Substrate: 0.02*M* acetylcholine chloride. At zero time addition of 7×10^{-6} g. eserine sulphate/ml. Ordinates: percentage of activity before addition of inhibitor. Abscissae: time in min. after addition of inhibitor.

(*c*) *Rate of dissociation of eserine-enzyme complex in presence of substrate*

When substrate is added to a mixture of enzyme and inhibitor the enzyme-inhibitor complex is decomposed according to the Michaelis-Menten equilibrium as follows:

$$EI \rightleftharpoons E + I \quad \text{(i)}$$
$$E + S \rightleftharpoons ES \quad \text{(ii)}$$

where E = free enzyme concentration; I = free inhibitor concentration; S = free substrate concentration; EI = inhibitor-enzyme complex; ES = enzyme–substrate complex, and the velocity of acetylcholine hydrolysis is dependent on the concentration of *ES*. Fig. 3 illustrates an experiment designed to examine the velocity of this change. The initial measured rate of hydrolysis was less than 12 per cent of an uninhibited control, and the shape of the curve would suggest that under these experimental conditions the control value was probably not much less than this, but it has not been found possible to obtain reliable figures in the first 1–2 minutes owing to insufficient lapse of time for the attainment of temperature equilibrium. Over the succeeding 50 min. the rate gradually rose until it reached an equilibrium rate which was 58 per cent of the control. The rate of attainment of equilibrium is dependent on the concentration of inhibitor and substrate employed—in particular low substrate concentrations prolong the period to equilibration and high substrate concentrations speed it up. The initial value obtained is, however, virtually independent of substrate concentration and is presumably dependent purely on the position of equilibrium in equation (i) before substrate competition has appreciably shifted it. The final equilibrium level of activity reached is, however, influenced by both inhibitor and substrate competitively.

It is clear that values obtained for cholinesterase inhibition will vary according to the arbitrary conditions selected. Equilibrium values will depend both on substrate concentration and the substrate employed. The higher the substrate concentration (Fig. 4) the smaller the degree of inhibition. *d*-Acetyl-β-methylcholine, which is employed as a specific substrate for " true " cholinesterase, has a lower affinity for the enzyme than acetylcholine, and in consequence eserine shows a greater equilibrium inhibition of cholinesterase with acetyl-β-methylcholine than with acetylcholine as substrate.

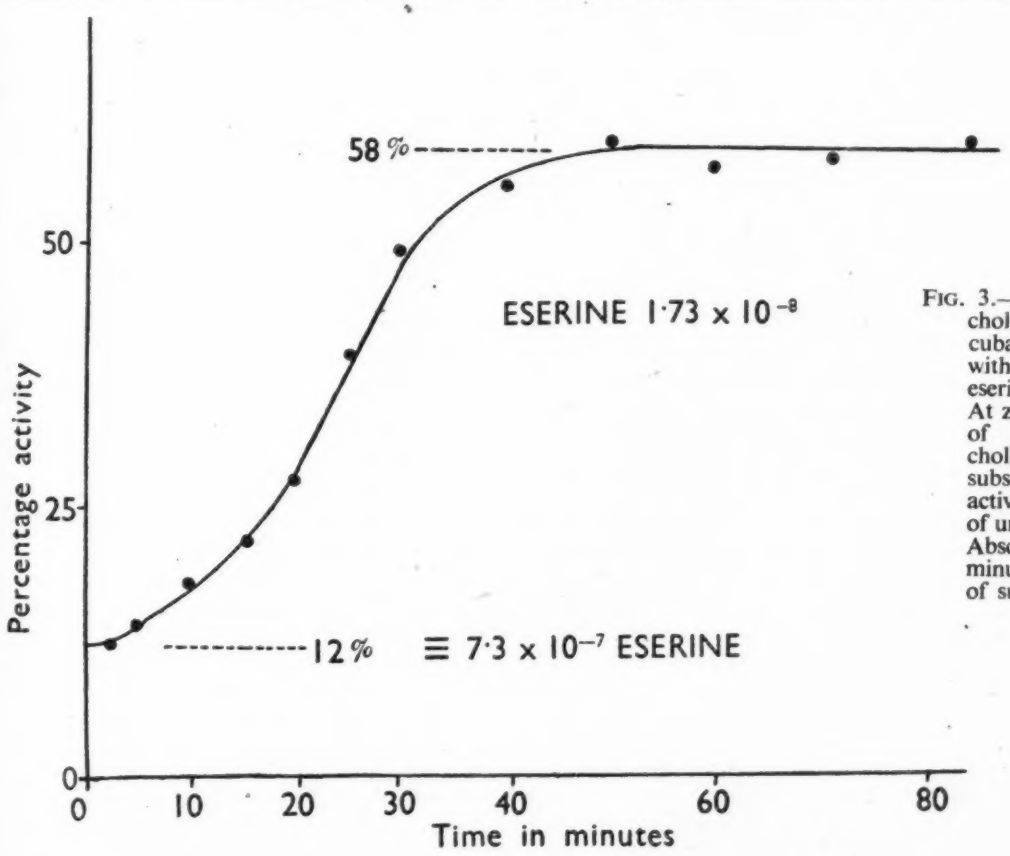


FIG. 3.—Human red cell cholinesterase. Incubated for 20 min. with 1.73×10^{-8} g. eserine sulphate/ml. At zero time addition of 0.005*M* acetylcholine chloride as substrate. Ordinates: activity as percentage of uninhibited control. Abscissae: time in minutes after addition of substrate.

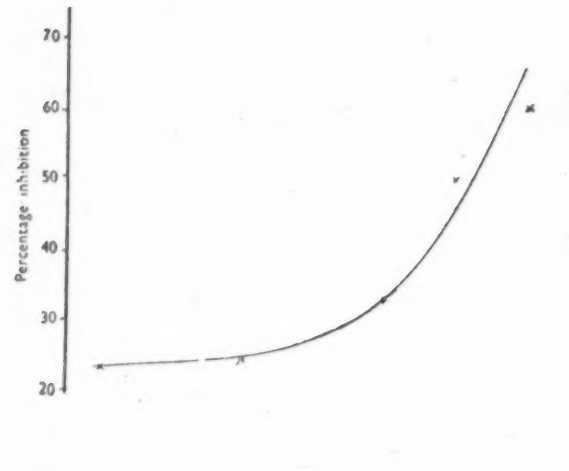


FIG. 4.—Human red cell cholinesterase. Substrate: acetylcholine chloride. *Lower curve:* Ordinates: velocity of acetylcholine hydrolysis in μl. CO_2 evolved/10 min. at equilibrium. Abscissae: molarity of acetylcholine. ●——● without addition of eserine. O- - - -O with 1.7×10^{-7} g. eserine sulphate/ml. *Upper curve:* Ordinates: percentage inhibition of cholinesterase by eserine. Abscissae: molarity of acetylcholine.

If, however, other than equilibrium conditions are considered the situation becomes chaotic. Many authors have reported results obtained in the first twenty or thirty minutes after adding substrate, but these data are influenced in such a complex way by both inhibitor and substrate concentration that their quantitative value is very doubtful. It would be of value to be able to determine the degree of non-competitive inhibition since this is unaffected by substrate concentration, but the experimental difficulties make the values only approximate, as they are necessarily obtained by extrapolation.

It is important to correlate these *in vitro* data with physiological events. The view that acetylcholine acts as a synaptic transmitter, accepted by most workers, requires that when no impulse is being transmitted at the synapse either no or an extremely small amount of acetylcholine leaks out of the cholinergic nerve terminals, whereas with the passage of an impulse a relatively large amount of acetylcholine is liberated and is present at the synaptic region for a period of milliseconds. This means that in the quiescent synapse we have essentially non-competitive conditions for cholinesterase inhibition. When acetylcholine liberation occurs its persistence is very brief compared with the long period required to establish competitive equilibrium, and is certainly insufficient to allow for more than a very small displacement of the inhibitor to occur. It would seem, therefore, that the equilibrium value discussed above grossly underestimates the effect of eserine under these conditions. The data in Fig. 3 show that the concentration of eserine employed (1.7×10^{-8}) produced only 42 per cent inhibition of the enzyme at equilibrium but produced at least 88 per cent inhibition under non-competitive conditions. To produce 88 per cent inhibition at equilibrium would require more than forty times as much eserine as was added. A further factor may come into play when the enzyme is greatly inhibited, since owing to the decreased rate of hydrolysis the acetylcholine concentration may rise sufficiently to cause some competitive decrease of the inhibition. In a prolonged nerve tetanus this displacement will increase during the course of the tetanus as acetylcholine accumulates and will reach an equilibrium value if the tetanus is of sufficient duration, but the position that this equilibrium is likely to reach cannot be estimated owing to our complete ignorance of the effective acetylcholine concentrations attained at the sites of cholinesterase activity in the synapse.

Another important aspect of these fast reactions has never been considered in connexion with cholinesterase, and that is the biphasic character of enzymic hydrolysis required by the Michaelis-Menten theory. The reaction occurs in two stages:

$$E + \text{Ac.Ch} \rightleftharpoons (E.\text{Ac.Ch}) \quad (1)$$
$$(E.\text{Ac.Ch}) \rightleftharpoons E + \text{Ac} + \text{Ch} \quad (2)$$

Stage (1) effectively removes acetylcholine without breaking it down and hence is a reaction of primary importance in disposing of acetylcholine. It is obvious that reaction (1) occurs in a shorter time than the overall reaction (1 + 2), and it is therefore of fundamental importance to know the relative rates of reactions 1 and 2. So far it has not been feasible to do this with cholinesterase, but we know from the direct measurements of Britton Chance (1943, 1948) on peroxidase and catalase that the first reaction ($E + S \rightleftharpoons ES$) may be as much as a hundred times faster than the second reaction and is mainly limited by probability considerations. If

this state of affairs can be applied to cholinesterase, it may be that the effective removal of part of the liberated acetylcholine can occur extremely rapidly. That this may be so is supported by the recent work of Eccles and MacFarlane (1949) on the effect of anticholinesterases on the frog end plate potential. They found that the upstroke of the end plate potential, which occurred in about 2 msec., rose higher and more steeply in the presence of anticholinesterases. Considering the low temperature at which they were working (16–18° C.) this time makes it probable that normally the formation of the enzyme-substrate complex is the most important factor in the initial rapid removal of acetylcholine, and that providing the enzyme capacity is adequate the actual hydrolysis of the complex is less important. Anticholinesterases will, of course, inhibit this reaction in a non-competitive manner.

Neostigmine behaves in a very similar way to eserine in all the equilibria so far considered, but other reversible inhibitors may behave differently. For instance, " 62C47 " (bis-trimethyl-aminophenyl-ethyl ketone diiodide, Glock and Mogey, 1948) reaches equilibrium more rapidly than eserine or neostigmine, so that the equilibrium under physiological conditions may be more competitive than with eserine or neostigmine. 62C47 is a less active inhibitor than eserine or neostigmine, and, as Goldstein (1944) has shown, the mass action velocities require that the less active a reversible inhibitor the more rapidly it should come into equilibrium, as is found experimentally.

Kinetics of TEPP Action

(*a*) *Rate of combination with the enzyme in the absence of substrate*

It is very easy to measure the rate of combination of TEPP with cholinesterase, because, as will be seen later, not only is the reaction almost completely irreversible *in vitro*, but the progress of the reaction is effectively blocked by addition of substrate. When cholinesterase was incubated with TEPP the enzyme slowly decreased in activity (Fig. 5) so that even after 40 min. the reaction was still proceeding, but at 2 hours little further inactivation was seen. By this time the amount of available TEPP must have fallen considerably as a result of hydrolysis, and this side reaction militates against the continued progress of the reaction. The initial rate of the reaction fits reasonably well the requirements for a bimolecular reaction. Thus if TEPP is added to the enzyme before addition of substrate it is evident that the degree of enzyme inhibition found is entirely dependent on the time of contact allowed before addition of substrate. This is in marked

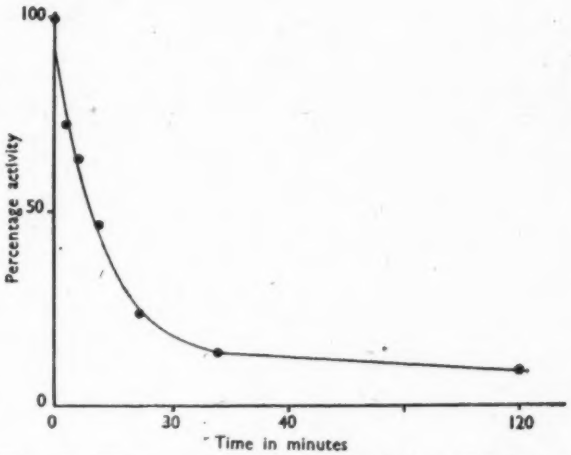


FIG. 5.—Human red cell cholinesterase. Substrate: 0.025*M* acetyl-β-methylcholine chloride. Incubation of 8×10^{-9} g. tetraethyl pyrophosphate/ml. with enzyme before addition of substrate. Ordinates: activity as percentage of control. Abscissae: period of incubation of inhibitor with enzyme before addition of substrate.

contrast to the state of affairs with eserine. In the experiment recorded in Fig. 1 20 min. contact between enzyme and TEPP was allowed. The choice of this time was arbitrary, but it is evident that for results to be comparable the same time interval must always be used. Actually, providing at least 10 min. contact is allowed, the error in estimating the potency of TEPP will be relatively small owing to the very steep concentration–inhibition relationship characteristic of this substance. It should be noted that DFP behaves very much in the same way as TEPP on the enzyme, but, as has also been found by Mackworth and Webb (1948), combines rather more slowly.

(*b*) *Combination of TEPP with enzyme in the presence of substrate*

If the experiment illustrated in Fig. 2 is repeated with TEPP as the inhibitor no detectable inhibition of cholinesterase results in the succeeding hour (Fig. 6, III), and even if TEPP and the substrate are added together very little inhibition results. This blocking by substrate is seen when either acetylcholine or acetyl-β-methylcholine is used as substrate for true cholinesterase. Even if the concentration of TEPP is increased to 10–100 times that usually required to produce inhibition, either no or only slowly developing inhibition occurs. With DFP and some other members of the group this is by no means so; for instance, with DFP some inhibition may gradually become apparent (Fig. 6, IV) even with concentrations (0.02–0.08 μg./ml.) producing considerable inhibition in

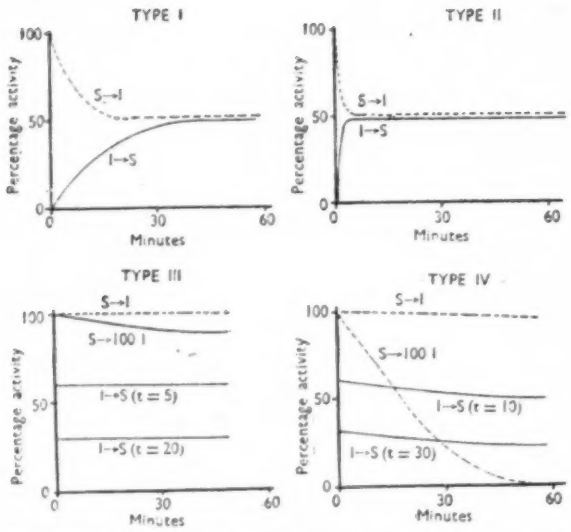


FIG. 6.—Diagrammatic representation of the four main classes of cholinesterase inhibitors studied. Ordinates: percentage activity of enzyme. Abscissae: time in minutes. S → I means substrate added before addition of inhibitor. S → 100 I substrate added before 100 times the amount of inhibitor needed to produce adequate inhibition in absence of substrate. I → S inhibitor added before substrate. The time of contact in minutes between inhibitor and enzyme, before addition of substrate, is shown in parentheses. I = typical results obtained eserine, neostigmine, and "Nu683"; II = results with "62C47." III = with TEPP. IV = with DFP.

the absence of substrate, and with higher concentrations (2–8 μg./ml.) inhibition appears quite rapidly despite the presence of substrate.

This blocking of TEPP inhibition by substrate may have some physiological significance. It may mean that at an active synapse the presence of acetylcholine may hinder the inhibitory effect of TEPP, so that the cholinesterase at that synapse is less inhibited than at a quiescent synapse. This blocking action of substrate for TEPP inhibition has been demonstrated clearly on the frog rectus abdominis preparation by Hobbiger (1949).

Blocking of TEPP inhibition by other inhibitors

The blocking action of substrate on TEPP is presumably due to competition for the same active groups on the enzyme, and it was therefore of interest to see whether cholinesterase inhibitors, which have a far greater affinity for cholinesterase than acetylcholine, would be able to block these groups as well. Fig. 7 illustrates a typical experiment of this kind. TEPP itself in a concentration of 1.33×10^{-8}, when incubated with the enzyme for 20 min., produced a 94 per cent inhibition (Fig. 7, curve *a*). Curve *b* shows the inhibition produced by increasing concentrations of eserine alone, whilst curve *c* shows the effect of incubating the enzyme with eserine for 20 min. followed by incubation for 20 min. with TEPP. It will be seen that at all concentrations of eserine some degree of protection of the enzyme results which increases with the concentration of eserine. Curve *d* is an expression of the percentage protection of the enzyme from inhibition by TEPP as a result of the prior contact of the enzyme with increasing concentrations of eserine; at a concentration of 10^{-8} eserine protects the enzyme to the extent of about 90 per cent from TEPP inhibition. It was also found that neostigmine, Nu 683, 62C47, carbachol, and choline were more or less efficient in blocking the action of TEPP, but that NaF, which by itself produces a 50 per cent inhibition of the enzyme at about 10^{-3} *M*, did not hinder TEPP inhibition and was in fact additive to it. It is easy to test rapidly whether a cholinesterase inhibitor blocks the action of TEPP by using a concentration of the reversible inhibitor that produces about 50 per cent inhibition and a concentration of TEPP that will produce 95–98 per cent inhibition in 20 min. If the substance is active as a protective agent the total inhibition will lie between 50 and 90 per cent. Koelle (1946) also showed that eserine, neostigmine, and to some extent carbachol could protect cholinesterase from irreversible inhibition by DFP. He was unable to show protection by acetylcholine or acetyl-β-methylcholine with his technique, which consisted of exposure of the enzyme to the protecting agent, followed by exposure to DFP for 30 minutes and subsequent dialysis against running water. His figures for inhibition of enzyme before dialysis, however, show clearly the effect of substrate described above. Koster (1946) has also shown that eserine has a protective action against DFP poisoning *in vivo*, and this has been confirmed by others.

The blocking of inhibitor action by substrate throws considerable light on the mechanism of the anticholinesterase action of the alkyl phosphate group of inhibitors. These agents might act in three ways: (*a*) a chemical reaction might occur between enzyme and inhibitor involving inactivation of both; (*b*) a chemical reaction might occur in which the effect of the inhibitor was catalytic and only the enzyme was changed; or (*c*) an initial reversible physical adsorption of inhibitor on the enzyme might occur followed by either (*a*) or (*b*). Nachmansohn, Rothenburg, and Feld (1947) have presented evidence that the inhibition of cholinesterase by DFP is biphasic, with an early reversible phase and a later irreversible phase. The data just presented support and extend this view. The

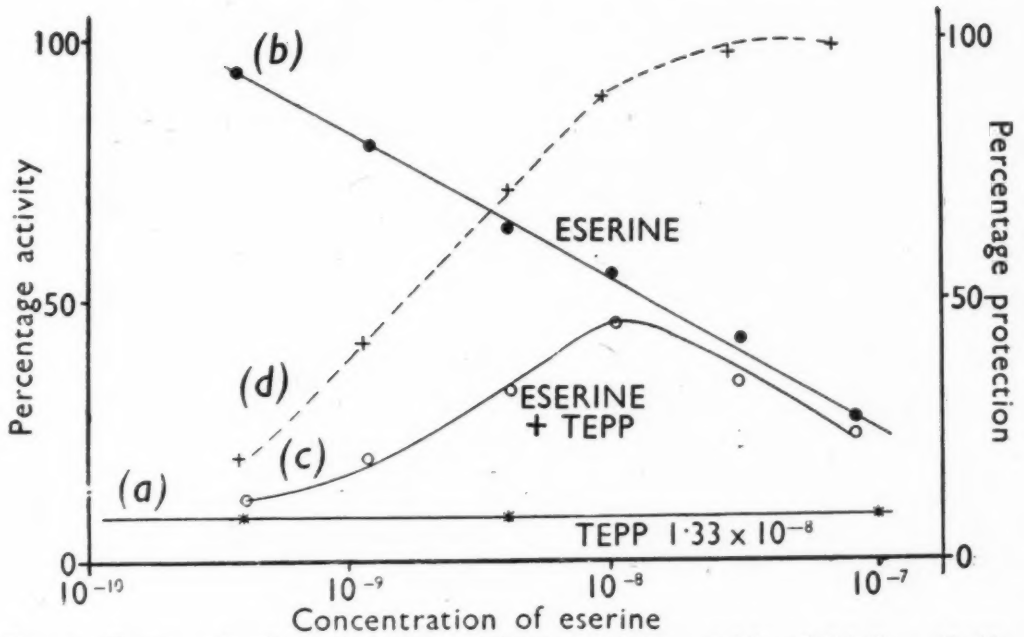


FIG. 7.—Human red cell cholinesterase. Substrate: 0.03*M* acetyl-β-methylcholine chloride. Ordinates (left): percentage activity of enzyme. Abscissae: concentration of added eserine sulphate. Curve (*a*): activity after incubation for 20 min. with 1.33×10^{-8} g. TEPP/ml. (*b*): activity after incubation for 20 min. with eserine sulphate. Equilibrium velocities. (*c*): incubation with eserine sulphate for 20 min. followed by 1.33×10^{-8} g. TEPP/ml. for 20 min. Equilibrium velocities. (*d*): percentage protection by eserine of the cholinesterase from inactivation by TEPP (right-hand ordinates).

blocking action of substrate is presumably due to the inability of TEPP to react with other than free enzyme centres. According to Michaelis and Menten (1913) the amount of free enzyme available is governed by the adsorption equilibrium:

$$E + S \rightleftharpoons ES$$

For a given substrate concentration the proportion of enzyme molecules uncombined (*E*) at any instant is fixed, and for the substrate concentrations used in our experiments is only a small proportion of the total available enzyme. If the rate of enzyme inactivation by alkyl phosphates depends entirely on the number of free enzyme centres, blocking by substrate should occur equally against DFP and TEPP, but this is not so. If, however, a reversible competitive combination is a preliminary to irreversible inactivation a second equilibrium will be involved:

$$E + I \rightleftharpoons EI$$

and the rate of inactivation of enzyme in the presence of substrate will be influenced by the dissociation constant of *EI* which will determine the amount of *EI* formed in the overall competitive reaction:

$$ES + I \rightleftharpoons EI + S$$

and just as with purely reversible inhibitors, a small dissociation constant for *EI* will favour its formation and a minimal blocking action by substrate, whereas if the dissociation constant is large—i.e., the affinity of enzyme for inhibitor is small—the blocking action by substrate will be considerable. The different ease of blocking with substrate can be explained if TEPP has a low adsorption affinity whereas that of DFP is high. The potency of an alkyl phosphate inhibitor thus depends on (*a*) the adsorption affinity, (*b*) the rapidity with which irreversible inactivation is produced in the enzyme-inhibitor adsorption complex. It remains to be established whether the second process is catalytic or involves the disappearance of inhibitor. Brauer (1948) has indicated that TEPP becomes no longer available when it is treated with cholinesterase. The quantitative nature of this change has been investigated as follows.

A constant amount of TEPP (final concentration 2.5×10^{-8}) was incubated with 0.02–0.5 mg. of purified cholinesterase per ml. at *p*H 7.2 and 37° C. for 30 min.; controls were simultaneously run containing either cholinesterase alone or 0.5, 1.0, and 2.5×10^{-8} TEPP alone. After 30 min. the *p*H was brought to 3.5 to 4.0 and the solution heated

at 96° C. for 3 min. This procedure has been found to destroy the residual cholinesterase activity completely whilst causing only very slight breakdown of TEPP. The solutions were then cooled and the *p*H adjusted to 7.2. One ml. of each solution was then incubated with 0.02 mg. of cholinesterase for 30 min. and the resultant activity of this indicator enzyme determined. The results obtained are illustrated in Fig. 8 (*a*), which shows that the more cholinesterase present initially the less the inhibition of the indicator enzyme. In order to determine whether these results could have been due merely to increasing protein concentration, the latter was held constant by adding to each tube a large excess of crystalline serum albumin; this procedure made no difference to the values obtained. Further evidence of the specificity of the reaction was obtained by the failure of either albumin alone or heat-denatured cholinesterase to decrease the available TEPP. These results fully confirm the experiments of

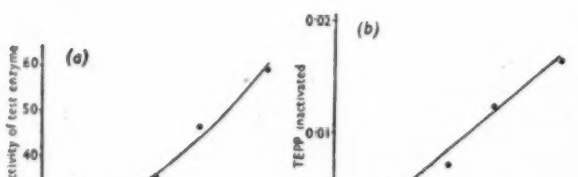
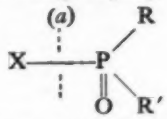


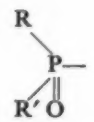
FIG. 8.—Purified bovine red cell cholinesterase. Substrate 0.025*M* acetylcholine chloride. (*a*) Ordinates: percentage activity of test enzyme. Abscissae: μg. cholinesterase added initially (experiment described in text). (*b*) Ordinates: μg. TEPP inactivated. Abscissae: μg. cholinesterase added initially.

Brauer (1948). Fig. 8 (*b*) relates the amount of cholinesterase added in the experiment of Fig. 8 (*a*) to the amount of TEPP that has become unavailable as estimated by inhibition of the indicator enzyme. It will be seen that the relationship is approximately linear, 1 mg. of cholinesterase removing about 0.034 μg. of TEPP under these conditions. A purely catalytic role of TEPP in the cholinesterase inactivation is thus excluded, and it is interesting to note that when the enzyme is heat-acid-denatured it loses its ability to combine with the alkyl phosphates as well as its enzymatic activity, and this argues further for the specificity of the chemical reaction involved.

If the biphasic interpretation of the action of the alkyl phosphates is correct, what is the nature of the secondary reaction? All the active members of the series contain the grouping



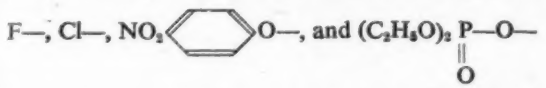
where the groups R and R′ may be a variety of structures and still retain activity, although the most active compounds are those in which R and R′ contain short alkyl chains. X is always a potentially acidic radicle so that the bond (*a*) is analogous to that in an acyl anhydride or halide and consequently rather less stable than the alkoxy or amido bonds joining R and R′ to the phosphorus. In view of this basic unit the most likely chemical change is a phosphorylation of the enzyme transferring the



radical to some polar grouping on the enzyme.

Most of the really active inhibitors are only weak phosphorylating agents, and in general increasing reactivity of the bond (*a*) decreases anticholinesterase activity; thus di*iso*propylchlorophosphonate is a much more active phosphorylator than di*iso*propylfluorophosphonate and yet is far less active as a cholinesterase inhibitor; in the series dimethyl-, diethyl-, and di*iso*propyl-fluorophosphonates the reactivity of bond (*a*), assessed by ease of hydrolysis, decreases in that order, yet the anticholinesterase activity increases in the same direction (Mackworth and Webb, 1948; Mazur, 1946). This is not always true; for instance, in the series of tetra-alkyl pyrophosphate derivatives in which one or both (P = O) groups are replaced by (P = S) the chemical reactivity falls as well as the anticholinesterase acitivity. It would appear that there is an optimum reactivity in the bond (*a*) which may be explained in the following way. Provided that the adsorptive affinity for the enzyme remains unchanged, if bond (*a*) is made more reactive the rate of reaction with the enzyme will be increased, but the rate of non-specific actions such as phosphorylation of random amino, hydroxyl, or phenolic groups in the protein may also increase, as will hydrolysis by water molecules. These side reactions will divert some of the active agent—an important matter with substances effective at very low concentrations. In order to explain the selectivity of the alkyl phosphates it is reasonable to assume that the adsorption complex of enzyme and alkyl phosphate introduces strain in bond (*a*) and thus facilitates phosphorylation by these weak acylating agents.

There are two alternative chemical reactions that may be considered briefly. The group X may combine with the enzyme; this is improbable because of the variety of chemical groups that are active in this part of the molecule; examples are:



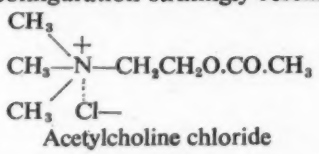
These groups differ greatly in chemical properties, and a mechanism which involves that they should all be highly active is clearly less probable than the relatively homogeneous mechanism suggested here. It is just possible that an alkylation is involved:

transferring R or R′,

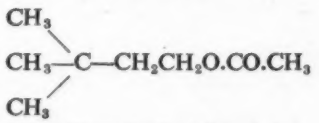
but these bonds are very stable to acid and alkaline hydrolysis, unlike bond (*a*). Further R and R′ may be alkoxy or dialkylamino without a very large change in activity and yet the groups to be transferred are vastly different in chemical potentialities; finally dialkoxy-, alkyl-, or aryl- phosphonates which have similar ester bonds but no anhydride structure are quite inert as cholinesterase inhibitors.

Brauer (1948) rejected the phosphorylation hypothesis on the basis of experiments with HETP (a mixture of alkyl phosphates obtained by heating ethyl phosphate with P_2O_5 or $POCl_3$, whose main active constituent is TEPP) labelled with P^{32}, in which he was unable to demonstrate association of the labelled phosphorus with the enzyme. His data, however, show that his preparation of labelled HETP contained only about 0.1 per cent of material active against cholinesterase; the association of this small amount of material with the enzyme would not be detectable under his experimental conditions. Michel and Krop (1949) have recently carried out a model experiment of this kind using DFP labelled with P^{32} and electrophorus cholinesterase. They found that the P^{32} of labelled DFP was precipitated with the cholinesterase by trichloracetic acid and the amount precipitated was proportional to the amount of cholinesterase added and to the degree of cholinesterase inhibition; 1 mg. of completely inactivated cholinesterase contained the phosphorus from 0.1 μg. DFP, and when allowance is made for the difference of enzyme activity and the molecular weights of DFP and TEPP this figure is very close to the one we obtained for TEPP and red cell cholinesterase by the indirect method. It can be taken therefore that members of the alkyl phosphate group inhibit cholinesterase by dialkylphosphorylation of the enzyme.

In the past it has been assumed that both acetylcholine and cholinesterase inhibitors combine with cholinesterase by virtue of the positively charged nitrogen, and it has been difficult to fit the alkyl phosphates into this pattern since they are predominantly negatively charged molecules and have no basic nitrogen. Adams (1949), however, has found that the essential feature of a substrate for true cholinesterase is the presence of an acetyl group, and that 3:3-dimethylbutyl acetate, which in general configuration strikingly resembles acetylcholine and yet lacks the quaternary nitrogen, is split almost as fast as acetylcholine. This work strongly suggests that combination with the enzyme occurs through the negatively charged ester linkage which is, of course, present in the alkyl phosphates. It is well known that carbamyl groups are isosteric with acetyl groups and hence the activity of the carbamyl esters may be explained without recourse to the basic nitrogen. Bloch (1939) arguing in this way prepared the acetyl and *iso*butyryl esters of *m*-hydroxyphenyltrimethylammonium (the basic half of neostigmine) and found these esters were active anticholinesterases.



Acetylcholine chloride



3:3-Dimethylbutyl acetate

Another aspect of the problem is the stability of the inhibited complex. Whereas both DFP and TEPP ultimately form apparently irreversible complexes with cholinesterase *in vitro*, the effects of TEPP are much shorter in duration than those of DFP *in vivo*. If the radical transferred to the enzyme were the same, the rate of recovery from the action both *in vivo* and *in vitro* would be the same, and hence the difference in duration of action between DFP and TEPP may be due to the difference between the di*iso*propylphosphoryl and the diethylphosphoryl radicals. If this theory is correct, the cholinesterase inhibition produced by diethylfluorophosphonate should be similar in duration to that produced by TEPP, and conversely the inhibition produced by tetra*iso*propylpyrophosphate should be similar in duration to that of DFP.

In support of this hypothesis may be quoted the following comment from Saunders and Stacey (1948), who compared the actions of di*iso*propyl-, diethyl-,

and dimethyl-fluorophosphonates on the eye, " We observed as early as 1941 that the dimethyl and diethyl esters produced a far less intense miosis than the di*iso*propyl ester. The effects of the dimethyl compound wore off in a matter of hours whereas that of the diethyl compound usually lasted about 2 days. . . ." The di*iso*propyl ester produced effects lasting usually for 7 days. In the cat we (Burgen, Keele, and Slome, 1949) found that the miotic effect of TEPP lasted about 1–2 days. Experiments in progress in this laboratory have shown in agreement with this theory that di*iso*propyl phosphoryl esters of different types have very similar durations of action to DFP, whereas other diethylphosphoryl esters have resembled TEPP in duration.

The breakdown of the dialkylphosphoryl-enzyme may be simply a matter of slow uncatalysed hydrolysis, and if so the *iso*propyl group would be expected to have a retarding influence on the reaction; alternatively the breakdown may occur through the operation of a phosphotriesterase. Aldridge (1949) has discovered a widely distributed enzyme in animal tissues which will hydrolyse diethyl *p*-nitrophenyl-phosphate to *p*-nitrophenol and diethyl phosphoric acid, and perhaps this or a similar enzyme could split off the dialkylphosphoryl group from the cholinesterase and so restore its activity.

Summary

1. The kinetics of inhibition of cholinesterase by both reversible and irreversible inhibitors is considered.

2. Methods of estimation in common use greatly underestimate the physiological activity of the reversible cholinesterase inhibitors because of competition with substrate and the slowness with which equilibrium is attained.

3. The action of many of the alkyl phosphate group is markedly blocked by substrate and by the presence of reversible inhibitors. Owing to this blocking by substrate the activity of this group may be overestimated *in vitro*.

4. The evidence available suggests that the alkyl phosphates form weak adsorption complexes with cholinesterase and in this activated state dialkyl phosphorylation of the cholinesterase occurs to form a stable inactive substance. This inactive enzyme may perhaps be regenerated by hydrolysis of the dialkyl phosphoryl group the rate of which is dependent on the nature of the alkyl groups. If *iso*-propyl phosphoryl groups form more stable complexes with the enzyme than diethyl phosphoryl groups, this may explain the longer duration of action of the di*iso*propyl series *in vivo*.

I am very grateful to Dr. C. A. Keele and Professor F. Dickens for helpful discussion. I am indebted to Mr. B. Topley and Drs. G. A. Mogey and F. Bergel for gifts of cholinesterase inhibitors and to Dr. M. L. Tainter for very kindly sending me a generous amount of purified cholinesterase. A grant from the University of London Central Research Fund to Dr. Keele enabled us to purchase the apparatus used in this work.

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Brit. J. Pharmacol. (1949), **4**, 229.

THE PHARMACOLOGY OF THE METHYL AND BENZYL ESTERS OF γ-CROTONIC BETAINE (γ-CARBOXYALLYLTRIMETHYL-AMMONIUM CHLORIDE)

BY

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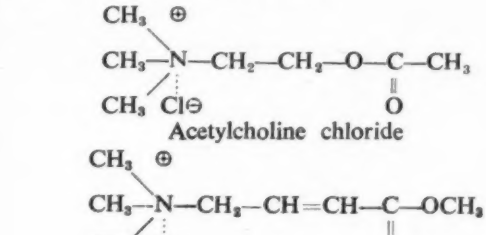
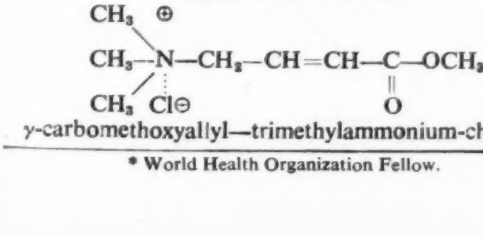
The effect of reversing the polarity of the ester linkage in acetylcholine homologues was first studied by Hunt and Renshaw (1926). These authors prepared aliphatic esters of betaine which they showed were parasympathomimetic agents with an activity of the order of one thousandth of that of acetylcholine. Betaine itself is pharmacologically almost inert and thus bears a relationship to its esters similar to that which choline bears to acetylcholine. The activity of the methyl ester of β-propionic betaine, the ester most closely isosteric with acetylcholine has not so far been reported. In 1928, however, a new betaine called γ-crotonic betaine (γ - carboxyallyltrimethyl - ammonium - chloride) was isolated from animal tissues by Linneweh (1928a, b). Strack and Försterling (1938, 1942) also isolated this betaine from mammalian muscle and prepared some of its esters. They found that whilst the betaine was almost inert pharmacologically, its methyl ester was a powerful parasympathomimetic drug when tested on the dorsal muscle of the leech, the frog rectus and heart and the mouse intestine. They also found that the ethyl ester had much weaker effects and in addition antagonized the effects of acetylcholine. The following paper confirms and extends these observations for the methyl ester and describes also the properties of the benzyl ester of this betaine. Chemically the structure of the methyl ester is quite similar to that of acetylcholine, as may be seen by the formulae:



Acetylcholine chloride



γ-carbomethoxyallyl—trimethylammonium-chloride

* World Health Organization Fellow.

Methods and Materials

Cats were anaesthetized with 60 mg. chloralose/kg. The tibialis anterior muscle was prepared for close intra-arterial injection as described by Brown (1938). The nictitating membrane response was recorded after section of the cervical sympathetic. The rabbit heart was perfused by the Langendorff method with Ringer-Locke solution. The rat phrenic nerve-diaphragm preparation (Bülbring, 1946) was set up in Tyrode solution containing 0.2 per cent (w/v) glucose and aerated with a 95 per cent O_2+5 per cent CO_2 mixture. Frog heart perfusions were carried out both by the sinus perfusion and the Straub methods. The chromodacryorrhoea response in rats was tested by the method described by Burgen (1949).

The crotonic betaine esters were prepared by the method of Bergel, Cohen, and Hindley (1949), and were made available to us as the methyl ester chloride (mol. wt. 182.5 ; m.p. 174° C.) and the benzyl ester iodide (mol. wt. 361 ; m.p. 147–149° C.). In the text these will be referred to as the methyl and benzyl esters and the activities are given by weight. In Table I the molar activities have been used.

Results

Effects on the anaesthetized cat

Methyl ester.—As little as 2 μg. of the methyl ester injected intravenously produced a small fall of blood pressure, unaccompanied by bradycardia. With increasing doses this effect increased, and when the dose reached 50 μg. a bradycardia accompanied the fall of blood pressure. As the dose was increased further this effect became more marked and was associated with a decrease in the tidal air. The fall of blood pressure and bradycardia were more prolonged than those given by an equivalent amount of acetylcholine (Fig. 1). Sometimes the respiratory depression was preceded by a transitory respiratory stimulation. This respiratory depression was unaffected by bilateral vagotomy or atropine, and respiration was shallow without the appearance of respiratory obstruction. It is probable that this effect was due to a depression of the respiratory centre or the

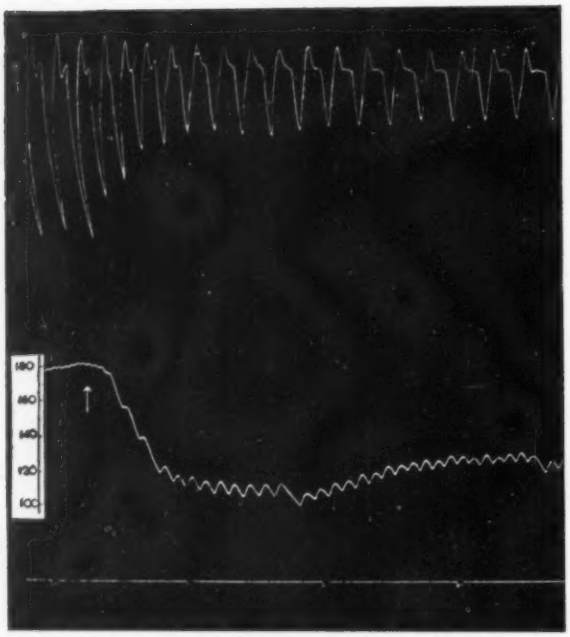


FIG. 1.—Cat, 2.4 kg., chloralose. Top tracing: respiration. Lower tracing: carotid blood pressure. Time in 5 sec. At the arrow i.v. injection of 0.14 mg. methyl ester.

muscles of respiration. Other effects noted with the larger doses of methyl ester were profuse and prolonged salivation, diarrhoea, and micturition.

After an adequate dose of atropine (1–2 mg./kg.) the slowing of the heart was no longer seen and the depressor effect of the methyl ester (100–200 μg.) was converted into a rise of blood pressure greater than that given by an equal dose of acetylcholine (Fig. 2).

Benzyl ester.—No effects were seen with the benzyl ester until 50 μg. were given. A small pressor effect then usually appeared which increased with increasing dosage but sometimes the response was biphasic. Respiratory depression occurred after the injection of 1 mg. of the benzyl ester and was similar in character to that given by the methyl ester. At no dose level were salivation, diarrhoea or micturition observed.

In the anaesthetized rabbit similar effects were seen.

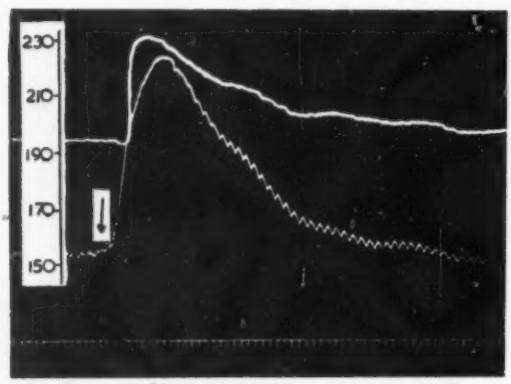


FIG. 2.—Cat, 2.4 kg., chloralose. Atropine, 2 mg./kg. Top tracing: nictitating membrane. Lower tracing: carotid blood pressure. Time in 5 sec. At arrow 0.2 mg. methyl ester intravenously.

Effects on the nictitating membrane

The methyl ester produced a contraction of the nictitating membrane when doses of 10 μg. or more were given intravenously (Fig. 2). The activity was similar to that of acetylcholine. With the benzyl ester, however, 0.5–1 mg. was needed to produce an effect and we did not succeed in producing a maximal contraction with the maximum tolerated dose. With both esters the response was quite long lasting.

Effects on the tibialis anterior preparation

Some of the effects obtained when the esters were injected intra-arterially into the tibialis anterior can be seen in Fig. 3; 20 μg. of the methyl ester produced a twitch similar in magnitude to that produced by 10 μg. of acetylcholine but followed by a transient depression of neuromuscular conduction. With 40 μg. of the methyl ester the twitch was somewhat larger, relatively to nerve stimulation, but was followed by a greater neuromuscular depression. Very similar effects

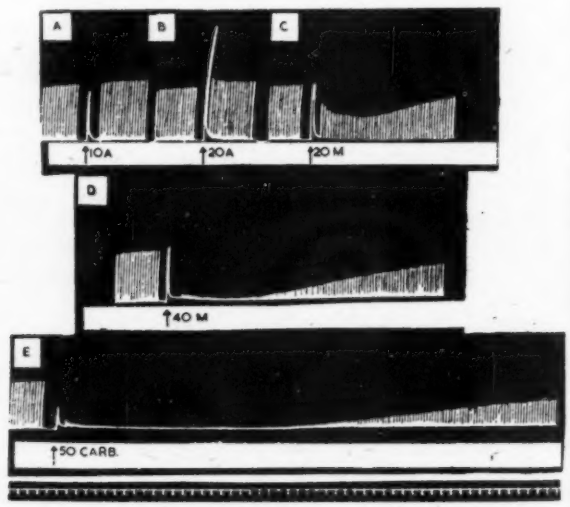


FIG. 3.—Cat, 2.1 kg., chloralose. Maximal motor nerve twitches of tibialis anterior muscle. Close intra-arterial injection of (A) 10 μg. acetylcholine; (B) 20 μg. acetylcholine; (C) 20 μg. methyl ester; (D) 40 μg. methyl ester; (E) 50 μg. carbamylcholine; volume of injection 0.5 ml.

were produced by 50 μg. carbamylcholine except that the twitch was smaller relative to the subsequent depression. 250 μg. of the benzyl ester produced a twitch similar to that given by 10 μg. acetylcholine, but was followed by a prolonged neuromuscular block.

Isolated preparations

Perfused rabbit heart.—The addition of 1 μg. of the methyl ester to the perfusion fluid exerted a negative inotropic effect about equal to that given by 0.5 μg. of acetylcholine or carbamylcholine. With 10 μg. this effect was increased and the heart rate slowed ; with 50 μg. the heart was temporarily arrested and this was followed by auriculoventricular dissociation. In these actions the methyl ester was about one quarter the activity of acetylcholine. The benzyl ester produced no depression of force or rate until 100 μg. were injected, when a transitory small depression occurred. After this dose, however, the response to acetylcholine was greatly decreased, and returned slowly over the succeeding 20–30 minutes.

Perfused frog heart.—The methyl ester depressed the force of contraction of the perfused frog heart in a concentration of about 0.01 μg./ml. With ten times this dose the heart was arrested. The activity relative to acetylcholine was about one tenth. The benzyl ester had no direct action on the heart even in a concentration of 1 mg/ml., but a well marked antagonism to acetylcholine was found with concentrations of the benzyl ester of 10 μg./ml. and higher.

Perfused vessels.—Rat and guinea-pig hind limbs and the rabbit ear vessels were perfused with Locke's solution at room temperature ; in all these preparations acetylcholine produced vasoconstriction. Both the methyl and the benzyl esters, given in about twice the dose of acetylcholine, produced a similar but more prolonged effect than that of acetylcholine. During the course of prolonged perfusions the sensitivity of these preparations increased equally for all three drugs.

Isolated rabbit duodenum and ileum.—The methyl ester (0.03–0.5 μg./ml.) produced a contraction of the longitudinal muscle of the rabbit duodenum and ileum similar in magnitude to that given by one-sixth to one-half the amount of acetylcholine (Fig. 4 *a*). The duration of the contraction was, however, longer than that given by acetylcholine, but resembled closely that produced by carbamylcholine. The benzyl ester produced a contraction in the freshly isolated intestine at a concentration of 1–2 μg./ml. After the gut had been isolated for one hour or more and especially if it was kept in the refrigerator overnight, the benzyl ester no longer increased the tonus, and with higher dosage a decrease in both the tonus and the spontaneous rhythm resulted. With the larger doses of the benzyl ester the motor effects of acetylcholine were antagonized, as may be seen in Fig. 4 *b*. When the intestine was set up as a Trendelenberg preparation, the peristalsis was completely inhibited by 10 μg. benzyl ester /ml. (Fig. 5 *b*).

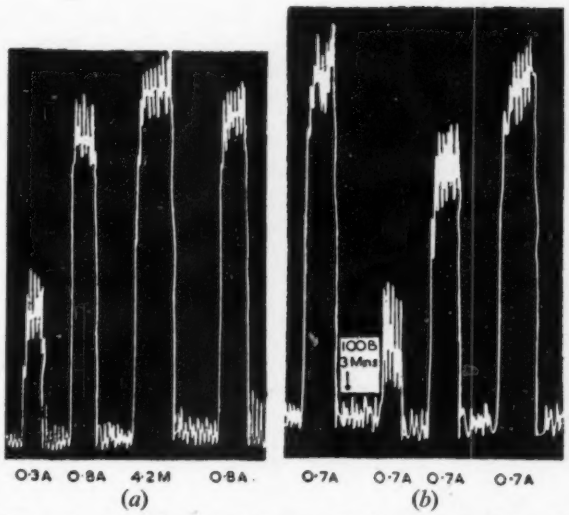


FIG. 4.—Rabbit duodenum. Bath volume 30 ml. (*a*) Comparative effect of acetylcholine (0.3 μg. and 0.8 μg.) and methyl ester (4.2 μg.). (*b*) Inhibition of the response to 0.7 μg. acetylcholine by 100 μg. benzyl ester.

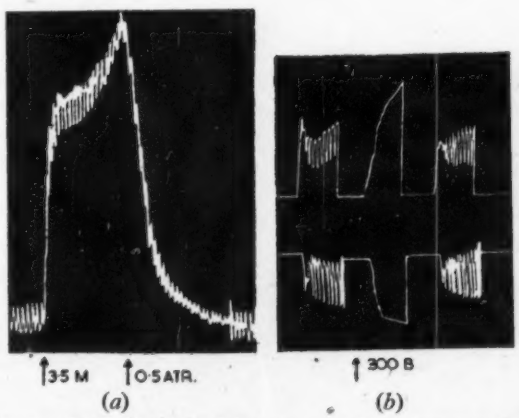


FIG. 5.—(*a*) Rabbit duodenum. Bath volume 30 ml. At first arrow addition of 3.5 μg. methyl ester which was promptly antagonized by 0.5 μg. atropine. (*b*) Guinea-pig ileum. Trendelenberg preparation. At arrow addition of 0.3 mg. benzyl ester for 3 minutes. Upper tracing: longitudinal muscle. Lower tracing: intestinal volume.

The motor effects of both the methyl and benzyl esters were readily antagonized by atropine (Fig. 5 *a*), and by adrenaline. The benzyl ester also antagonized the motor effects of the methyl ester.

Isolated guinea-pig ileum.—On the guinea-pig ileum the methyl ester produced a contraction in a concentration of 0.2–1 μg./ml. and was about one-half as active as acetylcholine. The benzyl ester had no direct effect on the intestine even in a concentration of 0.3 mg./ml., but at a concentration of 3 μg./ml. the response to acetylcholine was reduced by 50 per cent, and with higher concentrations could be abolished. The response to histamine was unaffected until 0.3 mg./ml. was added when it was depressed by about 10 per cent.

Phrenic nerve-diaphragm preparation.—Both the esters were fairly active neuromuscular blocking agents when tested on the phrenic nerve-diaphragm preparation. The methyl ester was about one-twentieth as active, and the benzyl ester was one-tenth as active as *d*-tubocurarine. Carbamylcholine was similar in potency to the methyl ester. Antagonism by neostigmine and by potassium chloride was much less marked with these esters than with *d*-tubocurarine. Paton and Zaimis (1949) report a similar lack of antagonism by neostigmine against the neuromuscular block produced by decamethonium iodide.

Frog rectus preparation.—On the normal unsensitized frog rectus preparation, both the methyl and the benzyl esters produced a contracture similar to that produced by acetylcholine, except that the latency was longer and the rate of development of the contracture slower. When the duration of the test was the usual one and one-half minutes the methyl ester was about one-third as active, and the benzyl ester one-fifteenth as active as acetylcholine (Fig. 6 *a*), whilst with a test lasting 10–15 minutes the relative potency of the crotonic esters was increased (Fig. 6 *b*). Whilst the absolute sensitivity of individual rectus muscles to the crotonic esters varied very little, the sensitivity to acetylcholine varied considerably, which accounts for the variation in relative potency.

Sensitization of the muscle with eserine or TEPP increased the response to acetylcholine on an average 40 times, but produced no change in the response to either the methyl or the benzyl ester. *d*-Tubocurarine antagonized both esters and acetylcholine about equally, but only the response to acetylcholine was restored by eserine.

Rat chromodacryorrhoea

Albino rats (c. 300 g. wt.) were used which gave no tear secretion after the injection of 250 μg. acetylcholine subcutaneously, but a well-marked red tear secretion to 500 μg. acetylcholine. The following results were obtained with the methyl ester.

20 μg.—no response.

50 μg.—pink tinging of the tears after 5–6 minutes.

100 μg.—profuse opaque red tears—some bradycardia.

1,000 μg.—profuse and very prolonged red tear secretion, heart block, profuse salivation, respiratory difficulties, spontaneous micturition and diarrhoea.

Doses of up to 20 mg. of the benzyl ester were ineffective in causing a red tear secretion.

In Table I the activities of the methyl and benzyl esters are summarized and compared on a molar basis with those of acetylcholine.

Hydrolysis by cholinesterases

The esters were incubated in 10^{-5} concentration at 37° C. either with a solution of lysed human red cells as a source of true cholinesterase or with human serum as a source of mixed esterases. The residual ester was estimated on the frog rectus preparation.

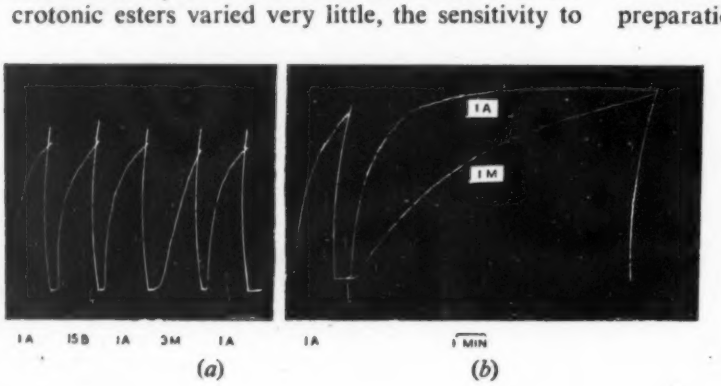


FIG. 6.—Frog rectus abdominis. (*a*) Comparison of 1 μg. acetylcholine with 15 μg. benzyl ester and 3 μg. methyl ester. Duration of test 90 sec. (*b*) Comparison of 1 μg. acetylcholine and 1 μg. methyl ester. Duration of test 10 min.

TABLE I

COMPARATIVE ACTIVITY OF THE METHYL AND BENZYL ESTERS

Preparation	Molar activity relative to acetylcholine = 100	
	Methyl ester	Benzyl ester
Frog rectus unsensitized...	30–100	15–50
,, ,, sensitized with eserine	2–5	0.5–2
Guinea pig ileum ...	50	0*
Rabbit duodenum† ...	15–50	5**
Perfused rabbit heart‡ ...	10–50	0*
Frog heart	10	0*
Cat blood pressure ...	8	1§
,, ,, ,, after atropine	250	20
Cat nictitating membrane	80	0.5
Cat tibialis ant. (twitch) ...	50	8
Rat diaphragm	1‖	5‖
Perfused vessels	50	20–100
Rat red tear secretion ...	1,000	0

* Blocks action of acetylcholine. ** Blocks action of acetylcholine and decreases intestinal tone (see text). § Biphasic or pressor response. ‖ Activity relative to *d*-tubocurarine chloride = 100. † Activity of carbamylcholine, 150. ‡ Activity of carbamylcholine, 100.

From the results given in Table II it will be seen that a preparation of true cholinesterase which hydrolysed acetylcholine very rapidly did not hydrolyse either ester. Serum did increase the breakdown of the benzyl ester, but at a rate far lower than for acetylcholine. The non-enzymatic rates of hydrolysis of the benzyl ester and of acetylcholine were measured by continuous titration over the range pH 4.5–9.5. The velocity constants were similar at the lower pH values, but activation by hydroxyl ions occurred sharply at pH 7.5 with the benzyl ester and at a slightly higher pH and less steeply with acetylcholine.

TABLE II

HYDROLYSIS OF METHYL AND BENZYL ESTERS BY CHOLINESTERASES

Ester	Enzyme	% Hydrolysed				
		10 min.	1 hr.	2 hrs.	3 hrs.	6 hrs.
Benzyl ester	No enzyme	—	5	—	12	24
	5% serum*	—	5	—	18	28
	1% R.B.C.*	—	−1	—	2	1
Methyl ester	No enzyme	0	—	2	—	—
	5% serum*	0	—	0	—	—
	1% R.B.C.*	3	—	3	—	—
Acetyl-choline	5% serum	97	—	100	—	—
	1% R.B.C.	99	—	100	—	—

* Corrected for non-enzymatic hydrolysis.

In experiments carried out in the Warburg apparatus it was found that 0.002 *M* methyl ester inhibited red cell cholinesterase 23 per cent (substrate: 0.025 *M* acetyl-β-methylcholine chloride) and inhibited serum pseudo cholinesterase 18 per cent (substrate: 0.007 *M* benzoylcholine chloride). These data show that both true and pseudo cholinesterases have an affinity for the methyl ester of the same order as that for acetyl-β-methyl choline and benzolycholine. It would seem that reversal of the ester link, whilst not reducing the affinity of cholinesterase for the methyl ester, prevents the enzyme-substrate complex breaking down into γ-crotonic betaine and methanol.

Summary

The *methyl* ester of γ-crotonic betaine is a very active parasympathomimetic drug. Both muscarinic and nicotinic types of action were found whose activity varied from one fiftieth to ten times that of acetylcholine according to the test preparation. The ester is not split by cholinesterase and hence has a more prolonged action than acetylcholine. The *benzyl* ester of γ-crotonic betaine has almost no muscarinic actions, but has some weak atropine like activity. Compared with the the methyl ester the nicotine actions are considerably weaker.

We are grateful to Dr. F. Bergel for placing a generous supply of these esters at our disposal.

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THE METHYLATION OF *NOR*ADRENALINE BY MINCED SUPRARENAL TISSUE

BY

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It has now been established that the pressor activity in extracts from the suprarenal glands is not due to the presence of adrenaline only but also to the presence of *nor*adrenaline. This has been shown for the pig's gland by Schümann (1948, 1949) and for the dog's gland by Bülbring and Burn (1949a) by biological estimation. That both substances are present in extracts of suprarenal glands from cattle was shown by v. Euler and Hamberg (1949) who compared the results obtained by biological methods with those obtained by paper chromatography (James, 1948) which they modified to yield quantitative results. Goldenberg, Faber, Aston, and Chargaff (1949) have also applied chemical and biological methods to show the presence of *nor*-adrenaline in commercial extracts of adrenal medulla from cattle and found that these contained 12–18 per cent *nor*adrenaline; one sample contained as much as 36 per cent. Tullar (1949) has isolated *l-nor*-adrenaline from such extracts.

The presence in the gland of the non-methylated compound may have two purposes. It may be an end-product which can be released into the blood-stream; it may also be the precursor for the synthesis of adrenaline by the gland.

There is recent evidence for the release of *nor*-adrenaline itself from the suprarenal medulla as was first suggested by Meier and Bein (1948). According to Holtz and Schümann (1949) the suprarenal gland releases a substance which is not adrenaline and is most probably *nor*adrenaline during the pressor reflex following clamping of the carotid arteries. Bülbring and Burn (1949b) showed that in the eviscerated cat stimulation of the splanchnic nerve to the suprarenal caused release of a mixture of adrenaline and *nor*adrenaline; this was shown by the fact that the pressor effect of splanchnic stimulation could be matched by an infusion of adrenaline, but the ratio of contractions of the two nictitating membranes, one denervated and one normal, could only be matched by infusing a mixture of adrenaline and *nor*adrenaline. In order to see whether the admixture of *nor*adrenaline was due to deficient methylation a number of cats were fed with methionine. However, in these cats also splanchnic stimulation produced a release of *nor*adrenaline with the adrenaline. The only difference observed was that the gradual decline in the proportion of adrenaline secreted during repeated splanchnic stimulation seemed to be absent in the animals fed on methionine.

This result suggested that the suprarenal gland, though normally releasing *nor*adrenaline to exert a function of its own, was also capable of methylating *nor*adrenaline in order to increase its store of adrenaline. The likelihood of this mechanism had been put forward by Blaschko (1939, 1942).

The present paper is concerned with the conversion of *nor*adrenaline to adrenaline by minced suprarenal glands *in vitro*.

METHOD

Dogs and cats were used. The suprarenal glands were obtained by three different procedures. The first was to anaesthetize the dog quickly with ether, bleed it and remove the gland with the shortest possible delay. The second was to anaesthetize the dog with ether, open the abdomen and remove the glands by operation while the dog was kept alive. The third was used in cats. Under ether anaesthesia the cord was cut, the brain destroyed and artificial respiration was given. In some experiments the right splanchnic nerve was dissected by retroperitoneal approach and was cut. Then the left splanchnic nerve was similarly dissected and stimulated. In other experiments both splanchnic nerves were stimulated simultaneously. After 30 minutes' stimulation the cat was bled out and the glands were removed with the shortest possible delay.

The suprarenals were dried with filter paper and weighed. They were then transferred into an icecold mortar, cut with scissors and ground with icecold saline, 1 c.c. per 0.05–0.1 g. gland. Of this suspension 2 c.c. were transferred to a test tube, 0.3 c.c. M/15 phosphate buffer was added, and in different experiments varying amounts of choline chloride, racemic *nor*adrenaline hydrochloride and adenosine triphosphate (ATP) were added. Each tube thus contained:

2.0 c.c. extract containing 0.1–0.2 g. gland.
0.3 c.c. M/15 phosphate buffer.
0.3–0.6 c.c. containing varying amounts of choline chloride.
0.3–0.6 c.c. containing varying amounts of *nor*adrenaline.
0.4 c.c. containing 8 mg. ATP.
3.3–3.9 c.c. total volume.

The weight of choline chloride added was always the same as that of racemic *nor*adrenaline hydrochloride, i.e., twice the amount of *l-nor*adrenaline. In those experiments in which no *nor*adrenaline was added the amount of choline added will be specifically stated. If any of the ingredients was omitted the corresponding volume of saline was added instead. For each experiment one or several control samples were prepared: the final procedure adopted was to add 0.3 c.c. buffer and 1 c.c. *N* HCl to 2 c.c. extract; this was quickly heated to boiling and immediately cooled. The same ingredients were added to this boiled control sample as to the one with which it was to be compared; the two were incubated alongside. The usual incubation time was 1 hour. After incubation 1 c.c. *N* HCl was added to each sample, though not to the control sample if acid had been added before incubation. The sample was then transferred to an Erlenmeyer flask. One washing with 2 c.c. buffer and two washings with 2 c.c. saline were also transferred into the Erlenmeyer flask. Each acidified sample was quickly heated to boiling point, cooled and made up to such a volume that 0.1 g. gland was present in 10 c.c. This solution was used for the assays.

In some experiments samples were incubated in Thunberg tubes evacuated and filled with nitrogen. The assay was carried out by the following methods.

The rat uterus method (de Jalon, Bayo, and de Jalon, 1945). This assay is based on the antagonism between adrenaline and acetylcholine. Constant contractions are evoked by constant doses of acetylcholine at constant intervals. The activity of the samples is estimated by the reduction in the size of contraction compared with that produced by a standard adrenaline solution. The preparation is almost invariably 100 times less sensitive to *nor*adrenaline than to adrenaline. It has been discussed in detail in a recent paper by Gaddum, Peart, and Vogt (1949) and I have used the same procedure. I am indebted to Professor Gaddum for the suggestion of using an apparatus according to Schild (1947) with a solution of carbachol instead of acetylcholine. In the later part of the experiments a simplified automatic apparatus has been used. I am indebted to Mr. O. B. Saxby for its construction.

The *frog heart*, in which the ratio N/A of activity of *nor*adrenaline to adrenaline is not as low as in the rat uterus, was used in one experiment only.

The *rabbit's duodenum* was used in 3 experiments. The ratio N/A for the rabbit's duodenum varied between 1 and 2. Adrenaline was never found to be the stronger.

The *spinal cat's blood pressure* was used in all except two experiments. The ratio N/A was very variable. In 3 out of 15 experiments it was less than 1 and only in 2 cats was it 2. The range was from 0.615 to 2.

The *cat's nictitating membrane*. This method was used in 11 out of 15 experiments. It has been described in detail in a recent paper by Bülbring and Burn (1949b) and is based on the fact that the denervated nictitating membrane becomes relatively much more sensitive to *nor*adrenaline than the normal membrane which is more sensitive to adrenaline. Thus the ratio of the size of contraction by the denervated membrane to that by the normal membrane is greater the larger the proportion of *nor*adrenaline in the mixture. By this means it is possible to test the activity of an extract in a spinal cat not only by the effect on the blood pressure, comparing it with adrenaline, but also on the nictitating membranes by comparing it with an equipressor dose of a known mixture of adrenaline and *nor*adrenaline.

For the calculation of the results obtained by different methods of different sensitivities a formula has already been used by Professor J. H. Gaddum (personal communication). I am grateful to Mr. J. St. L. Philpot for his advice in developing a formula as follows. Adrenaline was used as a standard throughout. Thus in a preparation of high sensitivity to adrenaline and of low sensitivity to *nor*adrenaline (e.g., rat uterus and frog heart) let 1 μg. *nor*adrenaline be equivalent to "*a*" μg. adrenaline. In a preparation of similar sensitivity to both let 1 μg. *nor*adrenaline be equivalent to "*b*" μg. adrenaline. Let the total activity on the rat uterus or frog heart be "*U*" (expressed as μg. adrenaline) and let the total activity on the rabbit's intestine or cat's blood pressure be "*C*" (expressed as μg. adrenaline). Then

$$U = A + aN$$
$$C = A + bN$$

where *A* is μg. adrenaline and *N* is μg. *nor*adrenaline. From these two equations the following formulae are derived:

$$A = \frac{bU - aC}{b - a}$$
$$N = \frac{C - U}{b - a}$$

These formulae were used for the calculations, of which two examples will be given.

The amount of *nor*adrenaline will be given throughout in terms of *l-nor*adrenaline.

Example 1

The estimation by the rat uterus method gave the following results for "*U*" in μg. adrenaline per c.c.

	Sample I	Sample II
1st strip	2.55	6.3
2nd strip	2.27	4.0
Mean "*U*"	2.41	5.15

The estimation on the cat's blood pressure gave for
Sample I "*C*" = 27.2 μg. adrenaline per c.c.
Sample II "*C*" = 25.8 μg. adrenaline per c.c.

The ration of activity $\frac{\textit{nor}\text{adrenaline}}{\text{adrenaline}}$ was on the rat's uterus "*a*" = 0.01, on the cat's blood pressure "*b*" = 1.8. From these results the values for adrenaline and *nor*adrenaline were calculated.

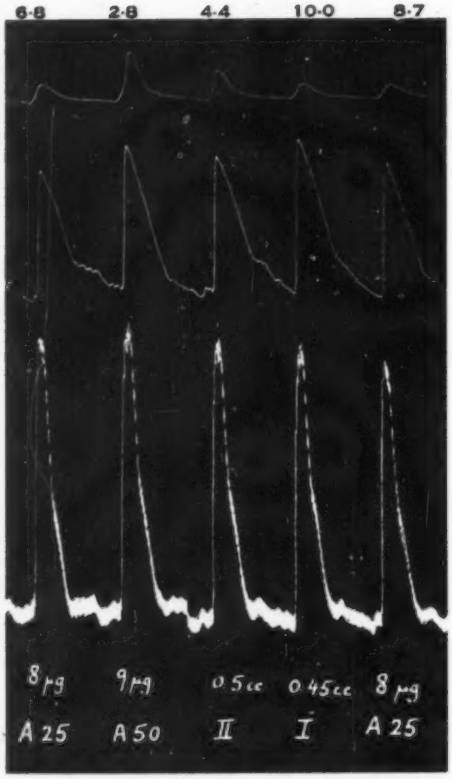

FIG. 1.—Assay of suprarenal extracts (Exp. 1) on spinal cat. Tracings are: arterial blood pressure (bottom), contractions of denervated (middle) and normal (top) nictitating membrane. Figures above are ratios of the size of contractions of the two membranes. I and II refer to samples in Exp. 1. "A" followed by a number indicates the percentage of adrenaline in a mixture of adrenaline and *nor*-adrenaline.

$$A_{\text{I}} = \frac{1.8 \times 2.41 - 0.01 \times 27.2}{1.8 - 0.01}$$

$$= \frac{4.35 - 0.27}{1.79} = 2.27\ \mu\text{g. adrenaline}$$

$$N_{\text{I}} = \frac{27.2 - 2.41}{1.8 - 0.01} = 13.9\ \mu\text{g. } \textit{nor}\text{adrenaline}$$

$$A_{\text{II}} = \frac{1.8 \times 5.15 - 0.01 \times 25.8}{1.8 - 0.01}$$

$$= \frac{9.30 - 0.25}{1.79} = 5.08\ \mu\text{g. adrenaline}$$

$$N_{\text{II}} = \frac{25.8 - 5.15}{1.8 - 0.01} = 11.5\ \mu\text{g. } \textit{nor}\text{adrenaline}$$

Another estimate was obtained by recording the contractions of the cat's nictitating membranes and comparing the extracts with mixtures containing varying proportions of adrenaline and *nor*adrenaline. On top of the tracing shown in Fig. 1 are given the ratios of the size of contractions of the denervated to those of the normal membrane. The ratio for Sample II was between those for two mixtures containing 25 per cent and 50 per cent adrenaline respectively. The ratio for Sample I was less than that for the mixture containing 25 per cent adrenaline. The actual percentages in the extracts were obtained graphically from a curve relating the ratio of contractions of the nictitating membranes to the percentage adrenaline present in the standard solution injected (see Bülbring and Burn, 1949b). When this was done, as shown in Fig. 2, the percentage for those injections of extract, shown in Fig. 1, were 20 per cent adrenaline in Sample I and 37 per cent adrenaline in Sample II. Though the pressor effects in this stage of the experiment were gradually diminishing it may be seen that 0.5 c.c. sample II was > 8 μg. mixture containing 25 per cent adrenaline and < 9 μg. mixture containing 50 per cent adrenaline.

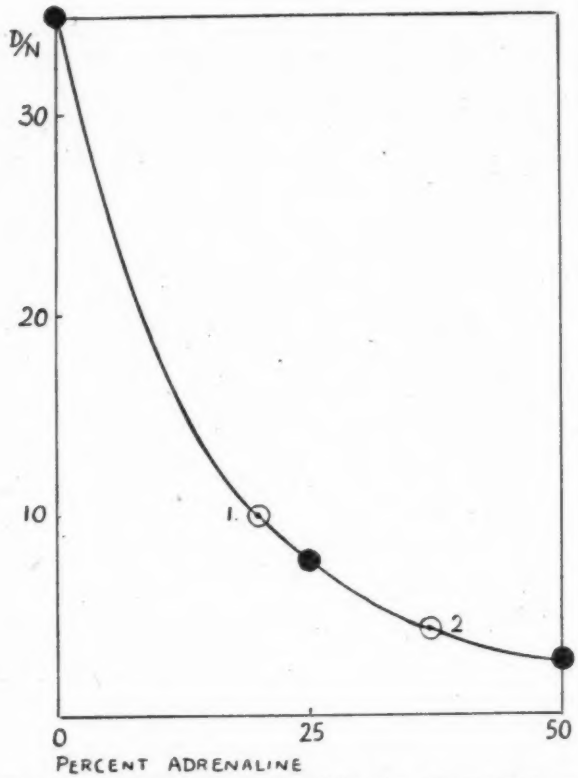

FIG. 2.—Curve relating ratio of the size of contractions of the denervated to the normal nictitating membrane (ordinates) and the percentage adrenaline in mixtures with *nor*adrenaline. The black circles represent effects obtained with standard solutions, white circles those obtained with suprarenal extracts.

$\therefore$ 1 c.c. sample II $\equiv$ 17 μg. mixture containing > 25 and < 50 per cent adrenaline.

0.45 c.c. sample I $\equiv$ 8 μg. mixture containing 25 per cent adrenaline

. . 1 c.c. sample I $\equiv$ 17.8 μg. mixture.

Estimations of this kind were repeated several times and the mean results were that

Sample I contained 21 per cent adrenaline in 17.76 μg. total activity.

Sample II contained 34 per cent adrenaline in 17.5 μg. total activity.

As the samples represented a 1 in 100 dilution of the suprarenal tissue the activity could be calculated as μg. per g. gland. This is shown in Table I.

TABLE I

ACTIVITY IN SUPRARENAL EXTRACTS

Sample	Estimate	Preparation used for estimation	μg. per g. gland		
			Adrenaline	*Noradrenaline*	Total
I	1st	Rat uterus and cat blood pressure	227	1,390	1,617
	2nd	Cat nictitating membrane and blood pressure	372	1,404	1,776
		Mean	300	1,397	1,697
II	1st	Rat uterus and cat blood pressure	508	1,150	1,670
	2nd	Cat nictitating membrane and blood pressure	596	1,154	1,750
		Mean	552	1,152	1,704

The agreement of the results obtained by the different methods was very close. It was so in 10 out of 15 experiments. Estimations of total activity did not differ from the mean figure of several combined assays by more than 10 per cent in 14 out of 15 experiments. The deviation from the mean figure for the adrenaline content was more than 20 per cent in 4 out of 15 experiments. One of these will be taken as the second example.

Example 2 (This was the experiment in which the difference between the two estimates was greatest.)

The assay by the rat uterus method gave widely differing results for "*U*" in μg. adrenaline per c.c.

	Sample I	Sample II	Sample III
1st strip ...	2.25	4.8	2.05
2nd strip ...	2.9	4.85	2.4
3rd strip ...	5.65	9.8	4.25
Mean ...	3.6	6.5	2.9

The assay on the cat's blood pressure gave the following results for "*C*":

1 c.c. Sample I equivalent to 48 μg. adrenaline
1 c.c. Sample II ,, ,, 34 μg. ,,
1 c.c. Sample III ,, ,, 44 μg. ,,

With "*a*" = 0.01 and "*b*" = 2.0 the activity in μg. per g. gland was calculated and is shown as the first estimate for each sample in Table II. The much weaker pressor activity of Sample II than that of Sample I, when compared with adrenaline, is shown in Fig. 3.

TABLE II

ACTIVITY IN SUPRARENAL EXTRACTS

Sample	Estimate	Preparation used for estimation	μg. per g. gland		
			Adrenaline	*Noradrenaline*	Total
I	1st	Rat uterus and cat blood pressure	340	2,230	2,570
	2nd	Cat nictitating membrane and blood pressure	840	1,560	2,400
		Mean	590	1,895	2,485
II	1st	Rat uterus and cat blood pressure	640	1,400	2,040
	2nd	Cat nictitating membrane and blood pressure	1,170	1,130	2,300
		Mean	905	1,265	2,170
III	1st	Rat uterus and cat blood pressure	270	2,080	2,350
	2nd	Cat nictitating membrane and blood pressure	550	1,650	2,200
		Mean	410	1,865	2,275

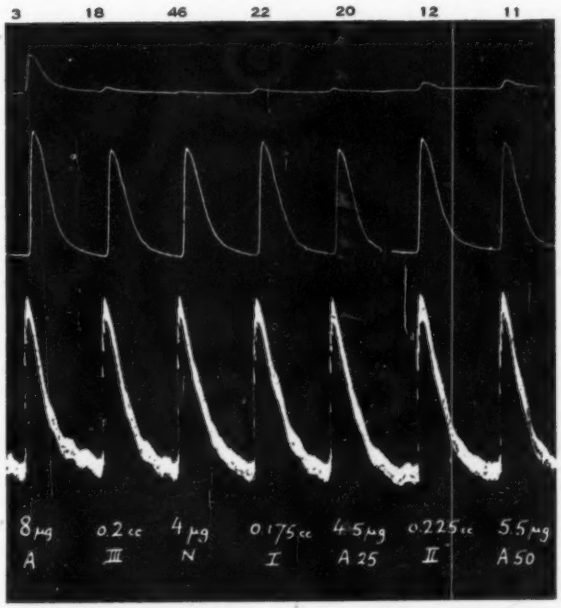

FIG. 3.—Assay of suprarenal extracts (Samples I, II, and III in Exp. 2) on spinal cat. Records as in Fig. 1.

8 μg. adrenaline = 4 μg. *nor*adrenaline
= 4.5 μg. mixture containing 25 per cent adr.
= 5.5 μg. mixture containing 50 per cent adr.
= 0.175 c.c. Sample I
= 0.225 c.c. Sample II
= 0.2 c.c. Sample III.

With these small doses, which were suitable for the assay of pressor activity, the normal nictitating membrane was scarcely affected. Larger doses had to be given for this purpose and part of the assay is illustrated in Fig. 4. By graphical determination from curves similar to that shown in Fig. 2 the proportion of adrenaline in the samples according to Figs. 3 and 4 were as follows:

	Sample I	Sample II	Sample III
Fig. 3	... 22 per cent	48 per cent	29 per cent
Fig. 4	... 38 ,, ,,	50 ,, ,,	16 ,, ,,

The mean figures of all estimations in this experiment are shown as the second estimates in Table II. The discrepancy between these and the other parallel assay is more than 100 per cent. However, the change in the three samples is in the same direction and of the same magnitude. This was so in every experiment without exception. No matter how large the absolute difference was between various assays for one sample (and it was never as large as in this sample), the relative difference between the samples was not affected; they always differed in the same direction. As there was no reason for assuming that either the assay combining results obtained on rat uterus and cat's blood pressure, or the assay in which extracts were compared with known mixtures on the nictitating membranes was more accurate than the other, the mean figure of both estimates was taken. The results were all converted to μg. per g. gland and are thus represented in the Tables.

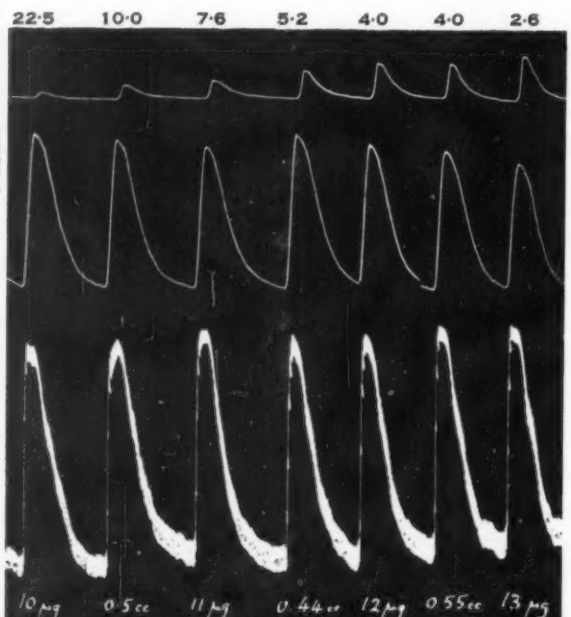

FIG. 4.—Assay (Exp. 2) continued. Records as in Fig. 1.

RESULTS

1. *The establishment of the control figure*

In order to observe a change in the proportion of adrenaline and *nor*adrenaline in a sample of suprarenal extract a control sample must be available which is subjected to the same conditions but in which enzyme activity has stopped. A comparison was therefore made on several occasions between extracts (*a*) acidified, boiled, incubated and not incubated, (*b*) non-acidified, boiled and then incubated, (*c*) the same, but with various additions. The results are given in Table III which shows the close agreement obtained by the combination of several biological methods. The total amount found in boiled control samples after one hour incubation did not differ by more than 6 per cent. from the samples which were not incubated. The agreement was close irrespective of whether they were acidified or not. A larger difference was found in Exp. 2 in the calculated total when a sample to which *nor*adrenaline had been added was compared with another without addition. Though the estimations of the amount of adrenaline and *nor*adrenaline in several samples of the same gland differed up to 20 per cent, the proportion of the two remained the same within 7 per cent. The largest differences were again found between estimations of samples to which *nor*adrenaline had been added and those without (Exps. 2 and 6), and also between samples incubated in O_2 and N_2 (Exps. 10 and 11). For this reason the control samples had the same additions and were subjected to the same conditions as those with which they were compared. The only exceptions are in experiments 4 and 5.

2. *The conversion of* noradrenaline *to adrenaline*

Eight experiments were performed on dogs' suprarenals, the results of which are summarized in Table IV. The first three glands were removed by operation under ether anaesthesia, the other glands were removed as quickly as possible while the dog was bled out. In Exp. 6, in which the dog was used as blood donor for a heart-lung preparation, the delay before removal of the glands was considerable; this may be the reason for no change taking place during incubation. In all the other experiments a conversion of *nor*adrenaline to adrenaline was observed. This can only be assumed if together with the increase in adrenaline a corresponding decrease in *nor*adrenaline is found. Thus the values for

TABLE III

DOGS' SUPRARENALS. COMPARISON OF CONTROL SAMPLES

Amounts of adrenaline (adr.) and *nor*adrenaline (*nor.*) are given in μg. per g. gland

No. of experiment	Acidified, boiled, no additions. *Not* incubated		*Not* acidified, boiled, ATP added. Incubated in O_2		*Not* acidified, boiled, ATP and choline added. Incubated in O_2		*Not* acidified, boiled, ATP, choline and *nor*adrenaline added. Incubated in O_2		Acidified, boiled, ATP, choline and *nor*adrenaline added. Incubated in O_2		Acidified, boiled, ATP, choline and *nor*adrenaline added. Incubated in N_2		Deviation of estimations from mean
	μg.	%	μg.	%	μg.	%	μg.	%	μg.	%	μg.	%	%
Adr.			645	44	640	41	655	48					−1.2, +1.3
(2) *Nor.*			810	56	905	59	690*	52					−13, +13
Total			1,455		1,545		1,345						−7.5, +7.2
Adr.	550	23			580	22.5							±2.7
(3) *Nor.*	1,875	77			2,000	77.5							±3.2
Total	2,425				2,580								±3.1
Adr.	635	63.5			643	65	680	68					−2.8, +4.2
(6) *Nor.*	365	36.5			353	35	320*	32					−7.5, +5.5
Total	1,000				996		1,000						±0.2
Adr.							1,073	47	900	42	1,120	49	−15, +5
(10) *Nor.*							1,210	53	1,270	58	1,175	51	−3.5, +4.3
Total							2,283		2,170		2,295		−3.5, +2.1
Adr.							1,050	37			1,200	41	±7.3
(11) *Nor.*							1,770	63			1,700	59	±2.0
Total							2,820				2,900		±1.4

* The amount of *nor*adrenaline added has been subtracted in order to make the sample comparable with the other samples of the same gland

"Percentage change in adrenaline content" are calculated by taking into account not only the amount of adrenaline estimated but also the amount of *nor*adrenaline. In Exp. 1 an increase of 588 μg. adrenaline was found, but the decrease of *nor*-adrenaline was only 506 μg. The conversion is thus not more than 506 μg. or an increase of 99 per cent on the original amount of 511 μg. adrenaline.

The presence of ATP was found to be essential for the methylation of *nor*adrenaline. In four experiments samples incubated with ATP showed an increase of adrenaline, whereas parallel samples without ATP showed a decrease, which in Exp. 10 was accompanied by a corresponding increase in *nor*adrenaline, indicating the possibility of demethylation. The addition in one experiment (No. 2) of ATP alone caused no change in the proportion of adrenaline to *nor*adrenaline.

The amount of *nor*adrenaline present in the samples had an important influence though it did not wholly govern the extent of the reaction. Exp. 2 offers a good example. Sample *a*, to which only ATP was added, showed no change. Sample *b*, to which both ATP and choline were added, showed an increase in adrenaline which was similar to that of sample *c* to which *nor*adrenaline was added as well. None of the added *nor*adrenaline seemed to have been used. In two experiments (2*b* and 3*a*) in which the original gland was rich in *nor*adrenaline, a conversion to adrenaline was observed though only ATP and choline were added. But in two other experiments in which the gland contained originally very little *nor*adrenaline (5*a*) or none (4*a*) no increase in adrenaline was observed. In the latter experiment there is a further indication for a conversion in the opposite direction. When *nor*adrenaline was added to the samples as well (4*b* and 5*c*) some increase in adrenaline content was observed in both experiments, though surprisingly little considering the large amount added (2,000 μg. per g. gland). In Exp. 5*b* a sample incubated with ATP and *nor*adrenaline, but omitting the choline, nevertheless methylated a similar amount to that methylated in the one to which choline had been added.

There were thus four points emerging from Table IV: (1) ATP was essential for the conversion; (2) conversion was possible if ATP and choline were added under certain conditions; (3) these conditions depended to some extent on the amount of *nor*-

TABLE IV

DOGS' SUPRARENALS

Amounts of adrenaline and *nor*adrenaline before and after incubation in μg. per g. gland

No. of Exp.	Original			Final			Change			Percentage change in adr. content	Additions before incubation		
	Adr.	*Nor.*	Total	Adr.	*Nor.*	Total	Adr.	*Nor.*	Total		ATP	Ch	*Nor.*
1	511	912	1,423	1,099	406	1,505	+588	−506	+82	+99	+	+	833
2*a*†	645	810	1,455	615	745	1,360	−30	−65	−95	−5	+	−	−
b	640	905	1,545	1,110	350	1,460	+470	−555	−85	+73	+	+	−
c	655	1,940	2,595	1,100	1,570	2,670	+445	−370	+75	+57	+	+	1,250
3*a*‡	580	2,000	2,580	750	1,750	2,500	+170	−250	−80	+29	+	+	−
b‡				450	1,970	2,420	−130	−30	−160	−17	−	+	−
4*a*‡	1,340	0	1,340	1,130	225	1,355	−210	+225	+15	−15	+	+	−
b	1,340	2,000*	3,340	1,515	1,695	3,210	+175	−305	−130	+13	+	+	2,000
5*a*‡	1,048	212	1,260	1,040	224	1,264	−8	+12	+4	0	+	+	−
b	1,048	2,212*	3,260	1,308	1,820	3,128	+260	−392	−132	+25	+	−	2,000
c				1,358	1,816	3,174	+310	−396	−86	+30	+	+	2,000
d				845	2,120	2,965	−203	−92	−295	−10	−	+	2,000
6	680	1,320	2,000	695	1,385	2,080	+15	+65	+80	+2	+	+	1,000
10*a*	983	1,238	2,221	1,170	1,100	2,270	+187	−138	+49	+15	+	+	500
b				753	1,465	2,218	−230	+227	−3	−23	−	+	500
11*a*	1,050	1,770	2,820	1,290	1,385	2,675	+240	−385	−145	+23	+	+	1,000
b				1,047	1,664	2,711	−3	−106	−109	0	−	+	1,000

* Values are calculated, not estimated. † Choline chloride addition was 2.5 mg. per g. gland.
‡ Choline chloride addition was 4.0 mg. per g. gland.

TABLE V

CATS' SUPRARENALS

s = splanchnic nerve stimulated, o = no stimulation; adr., *nor.*, and total in μg. per g. gland

No. of Exp.	Original			Final			Change			Percentage change in adrenaline content	Additions before incubation		
	Adr.	*Nor.*	Total	Adr.	*Nor.*	Total	Adr.	*Nor.*	Total		ATP	Ch	*Nor.*
7*a* s	360	3,685	4,045	480	3,408	3,888	+120	−277	−157	+33	+	+	3,000
b o	452	3,353	3,805	630	3,225	3,855	+178	−118	+50	+26	+	+	3,000
8*a*†s	485	870	1,355	765	450	1,215	+280	−420	−140	+58	+	+	−
b†o	617	363	980	598	372	970	−19	+9	−10	−2	+	+	−
9**a*s	377	1,173	1,550	447	1,271	1,718	+70	+98	+168	0	+	+	540
b o	1,144	1,745	2,889	1,164	1,817	2,981	+20	+72	+ 92	0	+	+	620
12*a* s	1,000	3,260	4,260	1,790	2,500	4,290	+790	−760	+30	+76	+	+	1,500
b s				820	3,400	4,220	−180	+140	−40	−14	−	+	1,500
13*a* s	422	1,650	2,072	615	1,465	2,080	+193	−185	+8	+44	+	+	1,500
b s				422	1,545	1,967	0	−105	−105	0	−	+	1,500
14*a* s	300	1,397	1,697	552	1,152	1,704	+252	−245	+7	+82	+	+	1,500
b s				287	1,242	1,529	−13	−155	−168	0	−	+	1,500
15*a* s	590	1,895	2,485	905	1,265	2,170	+315	−630	−315	+53	+	+	1,500
b s				410	1,865	2,275	−180	−30	−210	0	−	+	1,500

* The samples in this experiment were incubated in N_2. † Choline chloride addition was 1 mg. per g. gland.

adrenaline present but the reaction was probably limited by the amount of enzyme available; (4) the addition of the three ingredients—ATP, choline, and *nor*adrenaline—caused a conversion in 6 out of 7 samples.

A surprising result was that glands removed under the most favourable conditions converted less *nor*adrenaline into adrenaline than those removed during a prolonged operation. As it is known that there is an increased splanchnic discharge during ether anaesthesia and laparatomy, the effect of splanchnic stimulation was investigated.

Cats were used for this purpose and the results are summarized in Table V. In the first three experiments the splanchnic nerve to one gland had been stimulated but not to the other; both glands were incubated under the same conditions with the addition of ATP, choline, and varying amounts of *nor*adrenaline. Of the latter an excessively large amount was added in Exp. 7 and a conversion was observed in both glands, which was slightly more on the stimulated side. In Exp. 8 no *nor*adrenaline was added before incubation and a large difference was seen between the activity of the stimulated as compared with the non-stimulated side. Though the total activity of the stimulated gland was not less than that of the non-stimulated side (in fact it was 37 per cent higher) the proportion of adrenaline was much less. When both suprarenal extracts were incubated with ATP and choline the proportion on the stimulated side became the same as that of the other side in which it remained unchanged. This is shown in Table VI. In Exp. 9 (Table V) no

TABLE VI

SUPRARENAL EXTRACTS INCUBATED WITH ATP AND CHOLINE

Gland	Original Adr.: *nor*adr.	Final Adr.: *nor*adr.
Stimulated ...	36 : 64	63 : 37
Non-stimulated	63 : 37	62 : 38

change was observed on either side; though the amount of adrenaline was increased there was no corresponding decrease of *nor*adrenaline. The result is not clear because the total activity was found to be considerably more than that of the control samples. This was the only experiment in which the control samples were heated in a water-bath for 5 min. instead of bringing them to boiling point quickly. Also it was the only experiment, recorded in Table V, in which the samples were incubated in nitrogen. This point will be discussed later. On the other hand, it may have been that too small an amount of *nor*adrenaline was added for this gland.

In the last four experiments in Table V both splanchnic nerves were stimulated, both suprarenals were pooled and the extracts incubated with an amount of *nor*adrenaline which was expected to be well above the amount of adrenaline present in the gland after prolonged splanchnic stimulation. This expectation was fulfilled as in each experiment the amount of *nor*adrenaline was found to be 3–4 times that of adrenaline. In each experiment the increase in the amount of adrenaline after incubation was considerable, i.e., 76, 44, 82, and 53 per cent. However, in parallel samples to which no ATP had been added no such increase was observed. One of these samples (in Exp. 12) gave yet another indication of a conversion in the opposite direction.

The experiments on cats' suprarenals confirmed the observation that the presence of ATP is essential for the methylation of *nor*adrenaline. The glands in which the store of adrenaline had been depleted by previous splanchnic stimulation appeared to have a high enzyme activity.

It might be argued that any change in adrenaline should not be calculated as a percentage change of adrenaline initially present in the gland but rather of the *nor*adrenaline. This would, however, give misleading results as the initial amount of *nor*adrenaline depended largely on the very variable amounts added to different samples. For example, in sample 2*b* (Table IV) 470 μg. adrenaline was formed which is a conversion of 52 per cent of the initial 905 μg. *nor*adrenaline, while in sample 2*c* (of the same gland) the very similar formation of 445 μg. adrenaline represents only 23 per cent of the initial 1940 μg. *nor*adrenaline which was as high as this because 1,250 μg. had been added. Secondly, though splanchnic stimulation depleted the store of adrenaline the absolute figures of the initial content are on the whole similar to those of unstimulated glands and the highest absolute increase of 760 μg. occurred in a stimulated gland containing initially as much as 1,000 μg. adrenaline per g. (Exp. 12*a*, Table V).

Assuming that through the prolonged operative procedure in the first three dog experiments there occurred a certain amount of splanchnic stimulation the results may be summarized as in Table VII. (Exps. 6 and 9 have been omitted from this table as the absence of any change in either direction could probably be accounted for by faulty experimental procedure discussed above.) In glands depleted by splanchnic stimulation the increase in adrenaline content was larger than the variation found in control samples (see Table III). In 7 out of 10 samples it was more than 50 per cent and the mean

TABLE VII

EFFECT OF SPLANCHNIC STIMULATION

Stimulated glands			Non-stimulated glands		
No. of exp.	Percentage change in adrenaline content	Percentage change in total activity	No. of exp.	Percentage change in adrenaline content	Percentage change in total activity
DOGS					
1	+99	+ 5.8	4*b*	+13	−3.1
2*b*	+73	− 5.5	5*b*	+25	−3.2
2*c*	+57	+ 2.9	5*c*	+30	−2.6
3*a*	+29	− 3.1			
			10*a*	+15	+2.2
			11*a*	+23	−5.1
CATS					
7*a*	+33	− 3.9	7*b*	+26	−1.3
8*a*	+58	−10.4	8*b*	− 2	−1.2
12*a*	+76	+ 0.7			
13*a*	+44	+ 0.4			
14*a*	+82	+ 0.8			
15*a*	+53	−12.6			
Mean	+60.5		Mean	+18.6	

figure for all was an increase of 60.5 per cent from the original. On the other hand, in non-stimulated glands, no increase above 30 per cent was observed, only in 4 out of 8 experiments was it larger than the variation determined in control samples, and the mean figure for all was an increase of only 18.6 per cent. The variation in total activity was on an average no more than between several control samples. However, in two samples (Table VII; Exps. 8*a* and 15*a*) there was a loss of 10.4 and 12.6 per cent respectively. Nevertheless, in spite of this loss in total activity, the proportion of adrenaline was increased by 58 and 53 per cent respectively.

TABLE VIII

EFFECT OF ATP

No. of exp.	Suprarenal extracts incubated			
	With ATP		Without ATP	
	Percentage change in adrenaline content	Percentage change in total activity	Percentage change in adrenaline content	Percentage change in total activity
DOGS				
3	+29	− 3.1	−17	− 6.2
5	+30	− 2.6	−10	− 9.1
10	+15	+ 2.2	−23	− 0.1
11	+23	− 5.1	0	− 3.9
CATS				
12	+76	+ 0.7	−14	− 0.9
13	+44	+ 0.4	0	− 5.1
14	+82	+ 0.8	0	−10.0
15	+53	−12.6	0	− 8.5
Mean				
Dogs	+24.25		−12.5	
Cats	+63.75		− 3.5	

The experiments in which samples of the same gland were incubated with and without ATP are summarized in Table VIII. While there was always an increase of adrenaline when ATP was present there was either no change or a loss when ATP was absent. In Exp. 10, where the increase in adrenaline might not be significant it becomes more so when the loss of 23 per cent in the parallel sample containing no ATP is considered. On the average the loss in total activity does not exceed the variation in several control samples shown in Table III, but the samples containing no ATP were without exception found to be weaker than their control samples. The loss never exceeded 10 per cent.

3. *Experiments in aerobic and anaerobic conditions*

In four experiments a comparison was made between samples incubated in air and incubated in nitrogen. The results are shown in Table IX. It appears that methylation takes place anaerobically but not to the same extent as aerobically. Though the increase in the first two experiments is small and may not be significant, there is both in aerobic and anaerobic conditions a clear difference between samples incubated with and without ATP. The conclusion is drawn that anaerobic conditions are less favourable for the methylation of *nor*adrenaline and that this may be partly responsible for the negative result in Exp. 9 (Table V).

TABLE IX

EFFECT OF AEROBIC OR ANAEROBIC INCUBATION

No. of exp.	Percentage change in adrenaline content in suprarenal extracts incubated			
	In air		In nitrogen	
	With ATP	Without ATP	With ATP	Without ATP
10	+15	−23	+2	−22
11	+23	0	+17	−3
13	+44	0	+24	
14	+82	0	+58	

4. *The loss of total activity during prolonged incubation*

One experiment was carried out in which samples were incubated for different times. The loss was considerable, as shown in Table X. It may be seen

TABLE X

EFFECT OF DURATION OF INCUBATION

Dog's suprarenal, μg. per g. gland. Change during incubation

	Control			For 1 hour			For 1½ hours			For 2½ hours		
	%	μg.	%	μg.	Change	%	μg.	Change	%	μg.	Change
Adr. ...	22.5	580	30	750	+170	30	730	+150	33	690	+110
Nor. ...	77.5	2,000	70	1,750	−250	70	1,690	−310	67	1,410	−590
Total ...		2,580		2,500	−80		2,420	−160		2,100	−480

that nearly 20 per cent of the total activity was lost in 2½ hours. Though the proportion of adrenaline to *nor*adrenaline remained approximately the same up to 2½ hours the total loss of activity was then four times as much as the gain of adrenaline. This experiment indicates that the methylation of *nor*adrenaline proceeded quickly and was not limited by the time of incubation but by other factors.

Discussion

Blaschko's view (1939, 1942) that *nor*adrenaline is the precursor of adrenaline has been substantiated by the experimental results presented in this paper. *Nor*adrenaline and adrenaline are both present in suprarenal glands. They are both released, in varying proportions, from the gland during splanchnic stimulation, which might suggest that *nor*adrenaline is an end-product by itself. It has now been shown that it can be used by the suprarenal gland as precursor for the synthesis of adrenaline.

For the methylation of *nor*adrenaline by suprarenal extracts the presence of ATP appeared to be essential. No conversion took place in the absence of ATP. When *nor*adrenaline was added some samples converted a proportion of the added amount as well as the amount initially present in the gland. In other samples only a moderate degree of conversion took place in spite of a large surplus of *nor*adrenaline being available. Glands which had been subjected to prolonged splanchnic stimulation showed an increased capacity for the methylation of *nor*adrenaline, while glands removed as quickly as possible under the most favourable conditions converted relatively small amounts.

The conversion appeared to be completed within 1 hour (shorter periods have not been tested) during which the loss of total activity was negligible. If samples were, however, incubated for several hours a progressive loss in total activity was observed.

Holtz and Kroneberg (1948) proposed epinine rather than *nor*adrenaline as a precursor for adrenaline on the ground that *nor*adrenaline could be released from the suprarenal gland as an end-product. Recently (Holtz and Kroneberg, 1949) these authors have investigated the possibility of adrenaline synthesis by suprarenal extracts from phenylethylamine, tyramine, and oxytyramine. Incubation of extracts for several hours with the addition of any of these three amines produced an increase in pressor activity which the authors believe to be most probably due to the formation of adrenaline. It may, however, be due to various other reasons. Firstly, the control was neither acidified nor boiled. All the samples were shaken for several hours before the actual experiment was started in order to deplete the initial store. This depletion probably continued in the control samples during the period of the experiment while in samples to which the different amines were added the activity of amine oxidase might have been inhibited. Thus the stronger pressor activity could have been due not to a synthesis of adrenaline but to a prevention of its destruction.

On the other hand, a synthesis of adrenaline, either via epinine or via *nor*adrenaline, is considered by the authors. In the light of the experiments reported in this paper the synthesis of adrenaline is unlikely because no ATP was added. But the synthesis up to one stage before the end-product is possible. The synthesis of epinine is unlikely because of its feeble pressor activity. The synthesis of *nor*adrenaline is the most probable because it has a strong pressor activity and it may have been formed from oxytyramine during prolonged shaking in air. In their concluding remarks Holtz and Kroneberg (1949) correct their previous view by putting forward the theory that in the suprarenals the chief mechanism of adrenaline synthesis proceeds from oxytyramine via *nor*adrenaline to adrenaline. A side mechanism, which would enable the suprarenals to use phenylethylamine or tyramine for adrenaline synthesis, needs further analysis by suitable methods. Already in 1940 Vinet reported

experiments in which she incubated minced suprarenal tissue with hydroxytyramine and found a formation of what she believed to be adrenaline. For the estimation a colorimetric method was used, which, according to Euler does not distinguish between adrenaline and *nor*adrenaline. Thus in Vinet's experiments also a formation of *nor*adrenaline from hydroxytyramine might have taken place.

The methods employed for the assays in my experiments were far from accurate. It must, however, be emphasized that they agreed, without exception, in the estimation of the relative activity of different samples. The results are further strengthened by the use of known mixtures of adrenaline and *nor*adrenaline as standard solutions.

In a few samples the proportion of adrenaline to *nor*adrenaline was changed in the opposite direction indicating the possibility of the existence of a mechanism for the demethylation of adrenaline resulting in a formation of *nor*adrenaline. This has been suggested by Bacq and Fischer (1947) as one explanation for its presence in tissue extracts.

Summary

1. Suspensions of ground dogs' and cats' suprarenals are capable, during 1 hour's incubation at 37° C., of converting *nor*adrenaline to adrenaline.

2. The presence of ATP is essential for the methylation of *nor*adrenaline.

3. Glands removed after prolonged splanchnic stimulation have a higher methylating power than glands taken from a freshly killed animal.

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FORMATION OF ADRENALINE FROM *NOR*ADRENALINE IN THE PERFUSED SUPRARENAL GLAND

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Observations on the secretion of adrenaline from the perfused suprarenal gland of the dog have been described by Bülbring, Burn, and de Elío (1948). These experiments were carried out before the publication of work by various authors, Schümann (1948), Bülbring and Burn (1949a and b) and v. Euler and Hamberg (1949), showing that the suprarenal medulla contains *nor*adrenaline as well as adrenaline, and no tests were used which would have distinguished between the two substances. The numerous estimations were made with isolated loops of rabbit intestine, comparing the samples with adrenaline only. The main outcome was to show that the liberation of adrenaline-like material in the blood leaving the gland was modified by the presence of adrenaline in the perfusing blood. When this was very small in amount, stimulation of the splanchnic nerve liberated very little adrenaline-like material in the venous blood. When the experiment was performed with progressively larger amounts of adrenaline in the perfusing blood, stimulation of the splanchnic nerve liberated progressively larger amounts of adrenaline-like material until an optimum concentration for adrenaline in the perfusing blood was reached. A further increase of adrenaline in the perfusing blood beyond this optimum produced a steady decline in the amount of adrenaline-like substance liberated by splanchnic stimulation.

The work had, however, been begun with the object of studying the formation of adrenaline by the isolated gland, and in the experiments in which less than 1 μg. adrenaline per ml. was present in the perfusing blood, an increase in the amount of adrenaline-like material in the system was always observed. Meanwhile West (1947), working in the pharmacological laboratory in Edinburgh, had shown that the rat uterus was much more sensitive to adrenaline than to *nor*adrenaline. When used as described by de Jalon, Bayo, and de Jalon (1945) this test can be used to estimate adrenaline in a mixture of the two drugs (see Gaddum, Peart, and Vogt, 1949).

We therefore carried out a series of five perfusion experiments in which we estimated adrenaline and *nor*adrenaline separately throughout each experiment, and we now give a brief description of the results which were obtained.

METHODS

The experiments were performed as already described by Bülbring, Burn, and de Elío (1948), the right gland being first removed in order to determine the content of adrenaline and *nor*adrenaline per g.; this was assumed to be the content of the left gland at the beginning of the perfusion. All venous blood from the perfused gland was collected in a series of samples, and after the cells had been spun off, the plasma was tested. The left gland was tested at the end of the perfusion. By comparisons with adrenaline on the rabbit intestine a figure was obtained which we took as the sum of adrenaline + *nor*adrenaline. By comparisons with adrenaline on the rat uterus we obtained a figure which we took as the amount of adrenaline only.

RESULTS

Perfusion was carried out for periods which varied in different experiments from 2 hr. 7 min. to 3 hr. 25 min. The rate of flow through the gland diminished during the course of the perfusion. The following are the details of an experiment in which the flow was good throughout, starting at the rate of 12 c.c./min. and being 2.25 c.c./min. at the termination 2 hr. 19 min. later.

Experiment No. 1.

Right gland	Total	2,391 μg. or	1.65 mg./g.
	Adrenaline	1,162 μg	0.8 mg./g.
(by difference)	*Nor*adrenaline	1,229 μg.	0.85 mg./g.

The left gland weighed 1.6 g. Its active content at the beginning of the perfusion was assumed to be the same per g. as that of the right gland; hence

Left gland	Total	2,620 μg.
	Adrenaline	1,260 μg.
	*Nor*adrenaline	1,360 μg.

At the end of the perfusion the contents of the left gland were found to be

Total	2,062 μg.
Adrenaline	1,762 μg.
*Nor*adrenaline	300 μg.

During the perfusion the splanchnic nerve was stimulated continuously for 1 hr., and at the end of this period *nor*adrenaline was added to the perfusing blood so that the concentration present in terms of *l-nor*adrenaline was 5.4 μg./c.c. During the rest of the perfusion 144 c.c. of blood passed through the gland so that the amount of *nor*adrenaline added to the system in this way was 777 μg. In the venous blood from the perfused gland there was found

Total	1,073 μg.
Adrenaline	307 μg.
*Nor*adrenaline	766 μg.

The following balance could therefore be drawn

	At beginning	At end
Adrenaline in gland ..	1,260 μg.	1,762 μg.
,, in venous blood		307 μg.
*Nor*adrenaline in gland ..	1,360 μg.	300 μg.
,, added to blood ..	777 μg.	
,, in venous blood ..		766 μg.
	3,397 μg.	3,135 μg.

The figures for the totals at the beginning and at the end show fairly close agreement, there being a loss of less than 8 per cent. There was, however, a large loss of 1,071 μg. *nor*adrenaline and a large gain of 809 μg. adrenaline. The evidence supports the view that during the perfusion *nor*adrenaline was converted to adrenaline.

Of the four other experiments one was unsatisfactory because of the poor rate of flow through the gland. There was little change in the total contents of the system though the stimulation of the splanchnic nerve liberated both adrenaline and some *nor*adrenaline in the venous blood. The balance was as follows:

Experiment No. 2.

	At beginning	At end
Adrenaline in gland ..	1,215 μg.	415 μg.
,, in venous blood		821 μg.
*Nor*adrenaline in gland ..	0 μg.	85 μg.
,, added to blood ..	155 μg.	
,, in venous blood		244 μg.
	1,370 μg.	1,565 μg.

The total adrenaline was almost identical in amount at the end as at the beginning (1,236 μg. as compared with 1,215 μg.) though two-thirds of it appeared in the venous blood. The total *nor*adrenaline rose from 155 μg. to 329 μg. This change was an increase of 174 μg. on an initial total of 1,370 μg., that is to say, it was 12.7 per cent. The three remaining experiments were satisfactory. Since the results were similar to those in Exp. 1 they have been summarized together in Table I.

TABLE I

CHANGES OBSERVED DURING PERFUSIONS

Exp.	Total		Adrenaline		*Nor*adrenaline	
	μg.	%	μg.	%	μg.	%
1	−262	−8	+809	+64	- 1,071	−51
3	+235	+22	+315	+40	- 80	−26
4	+226	+16	+292	+29	- 66	−17
5	−503	−16	+315	+18	- 810	−61

The first point shown in the Table is that in each experiment there was a gain in adrenaline, and in each there was a loss in *nor*adrenaline. Even in the two experiments in which there was a net loss, there was nevertheless a gain in adrenaline, and in those in which there was a net gain this was exceeded by the gain in adrenaline. The changes have also been expressed as percentages of the amounts originally present. These percentages are large enough to show that the absolute changes are not so small as to be within the error of the biological methods used.

When each experiment is taken singly there is some correspondence between the loss of *nor*-adrenaline and the gain in adrenaline in experiments Nos. 1 and 5, but not in experiments Nos. 3 and 4, in which the recorded loss of *nor*adrenaline was small. In experiments 3 and 4 the glands contained adrenaline only, both at the beginning and at the end of perfusion, while in experiments 1 and 5 the change shown in Table II took place. In these

TABLE II

CONTENTS OF GLAND

	At beginning		At end	
No. of exp.	% Adr.	% *Nor.*	% Adr.	% *Nor.*
1	48	52	86	14
5	73	27	96	4

experiments both glands contained initially a considerable amount of *nor*adrenaline which at the end of the perfusion was very small. This finding also suggests that *nor*adrenaline was converted into adrenaline.

Discussion

The experiments furnish evidence that when the dog's suprarenal gland is isolated from all other tissues and is perfused with heparinized blood from a pump, *nor*adrenaline is methylated to form adrenaline in the course of the perfusion. A change consistent with this conversion was observed in each of four satisfactory experiments. In a fifth experiment in which the blood flow was poor there was no evidence of this change. The absence of conversion in this experiment added weight to the evidence of conversion in the others.

In each experiment the right suprarenal was analysed, without having been perfused, as a control, and in three of the five glands so examined *nor*adrenaline was found present as well as adrenaline. In these the *nor*adrenaline constituted respectively 14, 28, and 52 per cent of the total. In two of the glands, however, we found only adrenaline, and in glands taken from two other dogs also we found only adrenaline. For this reason *nor*adrenaline was added to the perfusing blood in the perfusion experiments.

These results agree with the evidence of Bülbring (1949) that minced suprarenal tissue transforms *nor*adrenaline to adrenaline. She observed that this process of methylation was much more active when the glands had first been depleted by a period of splanchnic stimulation. The splanchnic nerve was stimulated in experiments 1 and 3 of the perfusions, and the percentage increase in adrenaline was greater in these experiments than in the others in which the splanchnic nerve was not stimulated.

Summary

1. The dog's suprarenal gland may contain not only adrenaline, but also *nor*adrenaline. Out of seven glands three were found to contain both these substances.

2. When the gland was perfused with heparinized blood, *nor*adrenaline being added to this blood, an increase in the amount of adrenaline was observed coinciding with a decrease in the amount of *nor*adrenaline. The conversion of *nor*adrenaline to adrenaline is indicated.

We wish to thank Mr. H. W. Ling for help with the perfusions and Mr. J. R. Vane for carrying out some of the estimations.

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STUDIES IN THE CHEMOTHERAPY OF TUBERCULOSIS: PART V. THIOSEMICARBAZONES AND RELATED COMPOUNDS

BY

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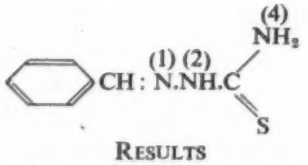
It has been reported (Domagk, Behnisch, Mietzsch, and Schmidt, 1946; Domagk, 1948) that some thiosemicarbazones of cyclic aldehydes and ketones show antituberculous activity *in vitro*. In some instances, activity *in vivo* was also demonstrated in experimental animals, and certain compounds have been used in the treatment of lupus and pulmonary tuberculosis (Moncorps and Kalkoff, 1947; Domagk, 1948; Kuhlmann, 1948) with results which are claimed to be encouraging. We have undertaken a systematic investigation of derivatives of thiosemicarbazide both *in vitro* and *in vivo*, using methods which have been described in earlier papers in this series (Martin, 1946; Hoggarth and Martin, 1948a). The compounds which have shown most interest are all thiosemicarbazones of monosubstituted benzaldehydes, the general formula of which may be written as follows:



RESULTS

The test method consisted of the infection of mice by the intravenous route, and their treatment by drugs administered orally twice daily by syringe and catheter at doses ranging downwards from the maximum tolerated. The results are presented in the form used in preceding papers.

(i) *Substitution in the aryl residue.*—Thiosemicarbazones of variously substituted benzaldehydes are listed in Tables I and II. The parent aldehydes in Table I carry one substituent. In some instances the same substituent has been placed in the three possible positions with respect to the thiosemicarbazone group. The compounds in Table II have two substituents in the aryl ring.

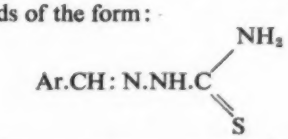
(ii) *Thiosemicarbazones of heterocyclical aldehydes.*—The results are given in Table III. Com-

TABLE I

ANTITUBERCULOUS TESTS ON SOME THIOSEMICARBAZONES OF MONOSUBSTITUTED BENZALDEHYDES

Doses given twice daily by syringe and catheter.

Compounds of the form:



No.	Ar	Dose (mg. per 20 g. mouse)	Increased mean survival time (days)	Increase required for significance (days)
5669	phenyl	0.25	+0.1	1.7
		0.5	+1.1	
		0.5	+2.7	3.0
5670	*p*-chlorophenyl	0.25	−0.3	1.7
		0.5	+2.1	
		0.5	+3.3	3.0
6056	*p*-nitrophenyl	0.5	+4.3	1.7
		1.0	+6.7	
		0.5	+7.2	2.2
		1.0	+8.7	
6267	*m*-nitrophenyl	0.1	+0.1	1.7
		0.25	−0.3	
5704	*o*-nitrophenyl	0.25	−0.3	1.7
		0.5	+0.9	
		1.0	−0.1	
6198	*p*-aminophenyl	1.0	+3.3	1.4
6087	*p*-methylaminophenyl	1.0	+4.1	1.6
		3.0	+2.4	
6147	*p*-ethylaminophenyl	0.25	+3.2	1.4
		0.5	+3.5	
5672	*p*-dimethylaminophenyl	1.0	+4.7	1.6
		1.5	+5.1	1.7
		2.0	+6.5	

TABLE I—contd.

No.	Ar	Dose (mg. per 20 g. mouse)	Increased mean survival time (days)	Increase required for significance (days)
6148	*p*-methylethyl-aminophenyl	0.25 0.5	+2.2 +2.9	1.4
5916	*p*-diethylamino-phenyl	0.25 0.5	+0.9 +3.4	1.9
5961	N-*p*-pyrroli-dinophenyl	0.25 0.5	0 +5.7	2.2
6473	*p*-di-β-chloro-ethylamino-phenyl	0.1 0.25	−0.4 +0.5	1.7
6478	*p*-ethyl-β-chloroethyl-aminophenyl	1.0	+2.2	1.2
6082	*p*-hydroxy-phenyl	10.0 10.0 10.0 20.0	+8.4 +8.1 +10.8 +11.2	2.8 1.3 2.0
5958	*o*-hydroxy-phenyl	5.0 10.0	+0.3 −0.8	1.4 1.6
6057	*p*-anisyl	2.5 5.0 5.0	+6.1 +8.4 +10.1	1.4 2.2 1.2
6083	*m*-anisyl	1.0 2.0 5.0	1.3 1.5 4.5	1.4
6253	*o*-anisyl	0.25 0.5	0 +0.7	1.4
6524	*p*-phenetidyl	2.0	+4.5	1.4
6772	*p-iso*propoxy-phenyl	1.0 2.0	+4.2 +5.6	1.4
6462	*p*-methylmer-captophenyl	0.25 0.5	+2.8 +4.7	1.4
8574	*p*-methylsul-phonylphenyl	5.0*	+10.0	3.3
8388	*p*-ethylsul-phonylphenyl	5.0 10.0 10.0	+12.7 +11.6 +9.8	2.7 1.5
8580	*p-n*-propylsul-phonylphenyl	10.0	+9.3	2.8
6463	*p*-thiocyano-phenyl	0.5 1.0	+4.2 +5.6	1.4

* This compound proved toxic at this dose, and ten of the twenty mice present originally in the treated group died in the first few days of the experiment.

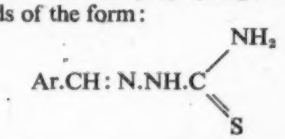
pound No. 6060 is the thiosemicarbazone corresponding to 5-nitrofurfural semicarbazone ("Furacin"). The latter was also tested under the same conditions (doses 0.5 and 1.0 mg. per 20 g. mouse) and was inactive.*

(iii) *Aliphatic thiosemicarbazones.*—Thiosemicarbazones derived from a few aliphatic aldehydes (heptaldehyde, cinnamaldehyde, *p*-dimethylamino-

TABLE II

ANTITUBERCULOUS TESTS ON SOME THIOSEMICARBAZONES OF DISUBSTITUTED BENZALDEHYDES

Doses given twice daily by syringe and catheter.
Compounds of the form:



No.	Ar	Dose (mg. per 20 g. mouse)	Increased mean survival time (days)	Increase required for significance (days)
5887	2: 4-dinitro-phenyl	0.5 1.0 2.0	+0.5 +0.8 +1.9	1.4 1.6
6465	3-nitro-4-dimethyl-aminophenyl	0.25 0.5	+0.1 0	1.0
6464	3-nitro-4-diethyl-aminophenyl	0.25 0.5	+0.4 +1.4	1.2
6475	2-chloro-4-di-β-chloroethyl-aminophenyl	2.0	+0.8	1.7
6468	4-ethylamino-*m*-tolyl	0.1 0.25	+1.1 +1.0	1.3
6476	4-di-β-chloro-ethylamino-*o*-tolyl	1.0 3.0	−1.9 +1.3	1.7
6222	4-hydroxy-*m*-anisyl	8.0	+0.6	1.4
6255	3: 4-dimethoxy-phenyl	1.0 2.0 2.0	+1.4 +2.9 +3.1	1.4
6266	4-ethoxy-*m*-anisyl	5.0	+5.3	1.4
6197	3: 4-methylene-dioxyphenyl	0.5 1.0	+1.9 +3.2	1.4

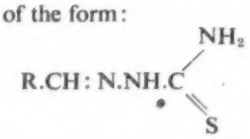
* It has been shown recently that "Furacin" has no therapeutic effect in tuberculous guinea-pigs (Wolinsky, E., Wetzel, V., and Steenken, W., 1949, *Proc. Soc. exp. Biol. Med.*, **70**, 483).

TABLE III

ANTITUBERCULOUS TESTS ON SOME THIOSEMICARBAZONES OF HETEROCYCLICAL ALDEHYDES

Doses given twice daily by syringe and catheter.

Compounds of the form:



No.	R	Dose (mg. per 20 g. mouse)	Increased mean survival time (days)	Increase required for significance (days)
6060	2-(5-nitrofur-furyl)	1.0 2.0	−1.6 −3.2	1.7
6116	2-quinolyl	2.5 5.0	+0.9 +1.1	1.2
6099	4-quinolyl	1.0 5.0	+1.6 +6.2	1.2

cinnamaldehyde, and dextrose) were all without any activity and results are not given in detail.

(iv) *Substitution in the thiosemicarbazone group.*—The hydrogen atoms of the thiosemicarbazide residue—i.e., those on nitrogen atoms N^2 and N^4, and also that on the sulphur atom in *iso*thiosemicarbazide (: N.N: C(SH)NH_2)—were replaced by alkyl groups, singly or in pairs. The parent aldehyde for most of these derivatives was *p*-dimethylaminobenzaldehyde or *p*-anisaldehyde. Results with these compounds are reported in Table IV *a* and *b*.

(v) *Ketone thiosemicarbazones.*—Some thiosemicarbazones derived from aliphatic and aromatic ketones (e.g., acetophenone, *p*-methoxyacetophenone, 5-diethylaminopentane-2-one) were found to be inactive.

(vi) *Benzaldehyde semicarbazones and benzalaminoguanidines.*—No activity was found in the semicarbazones of benzaldehyde, *p*-hydroxy, *p*-methoxy-, or *p*-dimethylaminobenzaldehyde, nor in the benzalaminoguanidines derived from the two last-named aldehydes.

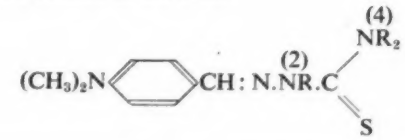
(vii) *Related compounds.*—The inactivity of the compounds mentioned in paragraph (vi) above indicated that the thioureido portion of the thiosemicarbazide group was essential. We have therefore examined other compounds in which the spatial relationship between an aryl nucleus and a thioureido group was similar to that in the benzaldehyde thiosemicarbazones. These compounds (21 in

TABLE IV

ANTITUBERCULOUS TESTS ON SOME SUBSTITUTED THIOSEMICARBAZIDE DERIVATIVES OF *p*-DIMETHYLAMINOBENZALDEHYDE

Doses given orally twice daily by syringe and catheter.

(*a*) Compounds of the form:

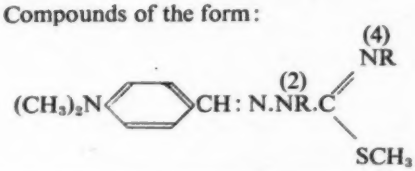


No.	Substituents on			Dose (mg. per 20 g. mouse)	Increased mean survival time (days)	Increase required for significance (days)
	N^2	N^4	N^4			
5873	H	CH_3	H	1.0 2.0	−0.2 +0.3	1.9
5874	H	C_2H_5	H	5.0	+1.6	1.9
5875	H	(*n*) C_3H_7	H	1.0 2.0	+0.7 +0.7	1.2
5876	H	(*iso*) C_3H_7	H	7.0	−0.8	1.9
5877	H	(*n*) C_4H_9	H	5.0 10.0 10.0	−1.5 −0.9 +0.1	2.2 1.9
5878*	H	(*iso*) C_4H_9	H	1.0 5.0 5.0 2.5 5.0	+2.5 +5.9 +5.7 +3.3 +8.5	2.2 1.9 2.2
6258*	H	(*sec*) C_4H_9	H	1.0 2.0	−0.2 −1.0	1.4
6067	CH_3	CH_3	H	1.0	+1.0	1.6
6068	CH_3	C_2H_5	H	0.5 1.0	+1.6 +0.5	1.6
6069	CH_3	(*iso*) C_3H_7	H	0.5	+0.4	1.6
6070	CH_3	(*n*) C_4H_9	H	10.0	+1.9	1.6
6290*	H	CH_3	CH_3	0.5 1.0	−0.8 −0.5	1.4

* Compounds analogous to Nos. 5878, 6258, and 6290, but prepared from *p*-anisaldehyde in place of *p*-dimethylaminobenzaldehyde, were inactive.

TABLE IV—contd.

(*b*) Compounds of the form:



No.	Substituents on		Dose (mg. per 20 g. mouse)	Increased mean survival time (days)	Increase required for significance (days)
	N^2	N^4			
6085	H	H	0.5 1.0	−0.3 +0.2	1.6
6080	H	CH_3	0.5 1.0	0 −0.7	1.4
6081	H	(*iso*) C_3H_7	1.0 2.0	−0.4 −0.4	1.5
6086	CH_3	CH_3	0.5 1.0	−0.7 +0.4	1.4

all) fall into the first six classes listed below. The last category (phenyl thioureas) represents the simplest possible combination of an aryl nucleus and a thioureido group. All these compounds were completely inactive.

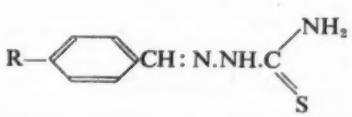
Ar.CH: N.NH.CS.NH_2
(Benzaldehyde thiosemicarbazones)
Ar.CO.NH.NH.CS.NH_2
1-Benzoylthiosemicarbazides
Ar.SO_2NH.NH.CS.NH_2
1-Benzenesulphonylthiosemicarbazides
Ar.CH: N.NH.CS.NH.N: CH.Ar
Dibenzalthiocarbhydrazides
Ar.NH.CS.NH.CS.NH_2
Phenyldithiobiurets
Ar.NH.C(NH).NH.CS.NH_2
Phenylguanylthioureas
Ar.CO.NH.NH.CS.NH.CO.Ar
1 : 4-Dibenzoylthiosemicarbazides
Ar.NH.CS.NH_2
Phenylthioureas
(Ar = an aryl residue usually with a *para* substituent which was either Cl, NO_2, HO, CH_3O, $(CH_3)_2N$, CH_3, or $NH_2.SO_2$.)

TABLE V

FURTHER ANTITUBERCULOUS TESTS ON THE MORE ACTIVE THIOSEMICARBAZONES OF MONOSUBSTITUTED BENZALDEHYDES

Drugs (mixed with powdered food) administered to groups of 20 mice for the first fourteen days of the test.

Compounds of the form :



No.	R	Estimated daily intake (mg.) of drug per 20 g. mouse	Observed mean survival time (days)	Increased mean survival time (days)	Increase required for significance (days)	First death (days)	Last death* (days)
5672	$N(CH_3)_2$	1.5 2.0	20.7 24.2	3.8 6.5	1.5 2.1	17 18	26 29(5)
6057	OCH_3	5.0 10.0 5.0 5.0	23.8 25.9 20.7 25.3	6.1 8.2 3.8 6.6	2.1 1.5 2.0	21 14 16 17	29(1) 29(3) 25(2) 33
6082	OH	10.0 20.0 2.0 5.0 10.0	29.6 30.0 22.4 27.2 30.5	10.8 11.2 2.8 7.6 10.9	2.0 3.0	19 20 18 20 22	35(2) 35(5) 30 31 33
8388	$SO_2C_2H_5$	2.0 5.0 10.0	32.7 34.8 35.4	13.1 15.2 15.8	3.0	25 26 28	40 50 50

* Figures in parentheses give number of survivors at the termination of the experiment. In all the above experiments all control mice were dead by the 22nd day except for a single mouse which survived to the 27th day in one test.

(viii) *Further comparisons of active compounds.*—Examination of Table I reveals that highest activity is shown by the thiosemicarbazones of *p*-hydroxybenzaldehyde (6082), *p*-anisaldehyde (6057), and the group of *p*-alkylsulphonylbenzaldehydes (8388, 8574, 8580). With all these compounds increases up to ten or twelve days in the mean survival time have been recorded, and it was felt desirable to compare these compounds directly one with another.

For this purpose we have preferred to administer the drugs mixed with the powdered food over a period of fourteen days, as previously described (Hoggarth and Martin, 1948b). We find that, on the whole, the effects observed with the two methods of administration (when the total daily intake of drug is the same) do not differ greatly from one another. The results are given in Table V.

The toxicities of the compounds in Table V are of interest in connexion with their therapeutic effects. In general the highest doses quoted in this table were the highest found to be tolerated without serious toxic effects by uninfected mice. We have not attempted to determine median lethal doses, but the observations recorded in Table VI were made.

TABLE VI

TOXIC EFFECTS OF THIOSEMICARBAZONES NOS. 5672, 6057, 6082, 8388

Drugs given by syringe and cathether twice daily to groups of twelve mice for three weeks.

No.	Dose (mg. per 20 g.)	Effect
5672	1.0	None
	2.0	Growth slightly retarded but no deaths
	4.0	Failure to gain weight (4 deaths)
6057	4.0	None
	6.0	Growth retarded but no deaths
	8.0	
	10.0	
6082	10.0	None
8388	4.0	None
	6.0	Growth slightly retarded but no deaths
	8.0	
	10.0	

From these limited observations, 6082 appears to be the least toxic, and in another experiment mice were given food containing 0.25% of this substance (estimated daily intake of drug 10 mg. per 20 g.) for 7 weeks without obvious toxic effects. When this concentration of 8388 was added to the food, the animals at first refused to eat it but later did so, without apparent harm, for 120 days.

Discussion

Our investigation of thiosemicarbazones and related compounds has shown that marked activity against an acute infection with *Mycobacterium tuberculosis* in mice is limited to the thiosemicarbazones of substituted benzaldehydes or heterocyclical aldehydes. Substitution of the hydrogen atoms of the thiosemicarbazide residue by alkyl groups usually abolishes activity, and the few thiosemicarbazones derived from alkyl-substituted thiosemicarbazides which are active are usually less so than the parent compound. Activity has not been found amongst the many closely related compounds examined. The relationship between activity and the type and position of substituents in the aromatic nucleus of benzaldehyde thiosemicarbazones may be inferred from Tables I and II. For highest activity a *para* substituent is necessary, though a few substituents in the *meta* position confer activity. High activity is found with alkylsulphonyl, nitro, amino, hydroxy, mercapto, and thiocyano groups in the *para* position, but only low activity with a *para* chloro group. Alkylation of the *para* hydroxy group does not result in significantly higher activity. Amongst the *p*-aminobenzaldehyde thiosemicarbazones the peak of activity is shown by the dimethylamino compound. We have examined three thiosemicarbazones of *p*-alkylsulphonyl benzaldehydes, all of which are highly active; the ethylsulphonyl compound is possibly the most active. The methylsulphonyl compound is more toxic than the other two. Introduction of a second substituent in the aryl residue usually reduces activity greatly. The activity of quinoline-4-aldehyde thiosemicarbazone is within the range of activity of the benzaldehyde thiosemicarbazones.

The response observed with the most active compounds of this type (Nos. 6057, 6082, 8388, 8574, and 8580) under the particular conditions of our routine test was better than that obtained with any other synthetic substance (including *p*-aminosalicylic acid) which we have yet examined. When the drugs were given mixed with the food for the first fourteen days only of the test, it has proved possible with most of these compounds to keep all treated animals alive until after the death of the last control animal. The treated animals did eventually die with marked tuberculous lesions of the lungs.

The activity *in vitro* of all the compounds mentioned above was determined by our usual method (Hoggarth and Martin, 1948a), but the results are

not given here in order to save space. We agree with the general statement of Domagk and his co-workers that the compounds with most activity *in vitro* in this series will inhibit growth of the tubercle bacillus at concentrations of 1 : 50,000–1 : 100,000. In our *in vitro* test we commonly find that the concentration at which growth is equal to controls is not less than 1/9 or 1/27 of the concentration for complete inhibition—e.g., No. 6082 will completely inhibit growth at 1 : 81,000 but has no inhibitory effect at 1 : 2,000,000. With certain compounds of this series—e.g., Nos. 6056, 6099, 6255, 6266, 8388—partial growth took place even at 1 : 1,000, the highest concentration tested, but inhibition of growth could be detected at a concentration of 1 : 729,000 or 1 : 2,000,000. We believe this unusually wide range of partial activity to be a real phenomenon, as it has been observed repeatedly with the substances mentioned and also occasionally with substances having no therapeutic action. Many compounds of high activity *in vitro* were devoid of activity *in vivo*.

The substances tested clinically by the German workers may be identified by the key given by Domagk (1947). These correspond to our numbers 8388, 6057, and the acetyl derivative of our number 6198. We find that the thiosemicarbazone of *p*-ethylsulphonylbenzaldehyde (8388) is superior in its therapeutic effect to any of the other compounds of its class which we have examined. This is a reflection of its marked persistence in the blood stream (Spinks, 1949).

Summary

Antituberculous activity in mice has been investigated in certain derivatives of thiosemicarbazide and related compounds. After a study of about one hundred such compounds, therapeutic activity has been found to be confined to thiosemicarbazones of substituted benzaldehydes and heterocyclical aldehydes.

Our thanks are due to Dr. N. Barton of these laboratories for samples of phenyldithiobiuret compounds originally prepared by him for antimalarial test.

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THE ESTIMATION OF SOME THIOSEMICARBAZONES AND THEIR BLOOD CONCENTRATIONS IN EXPERIMENTAL ANIMALS

BY

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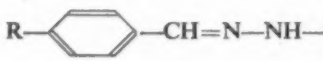
Thiosemicarbazones of some aromatic aldehydes have been shown to possess appreciable antituberculous activity (Domagk, Behnisch, Mietzsch, and Schmidt, 1946; Domagk, 1948; Hoggarth, Martin, Storey, and Young, 1949). Among the compounds described by Hoggarth *et al.* (1949) the most promising were *p*-anisaldehyde thiosemicarbazone (6057; I, R = CH_3O), *p*-hydroxybenzaldehyde semicarbazone (6082; I, R = OH) and *p*-ethylsulphonylbenzaldehyde semicarbazone (8388; I, R = $C_2H_5SO_2$). The methods described here were



6057: R = CH_3O (I)
6082: R = OH
8388; R = $C_2H_5SO_2$

developed so that the absorption and excretion of these three compounds could be studied in relation to their therapeutic effects in mice.

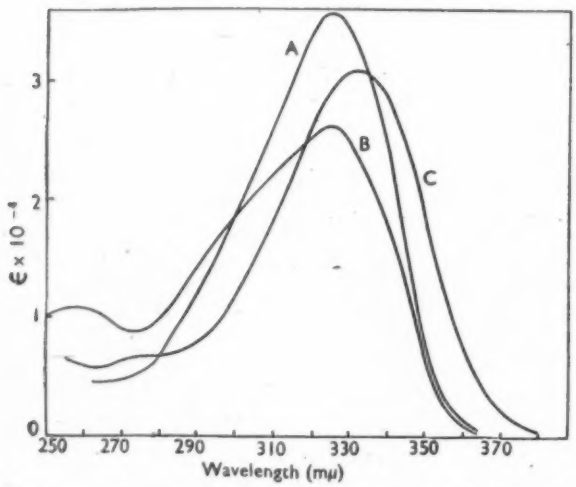


FIG. 1.—Absorption spectra of 6057 (A), 6082 (B) and 8388 (C) in chloroform. Ordinates: $\epsilon \times 10^{-4}$; abscissae: wavelength (mμ).

EXPERIMENTAL SECTION

It is known that certain unsaturated thiosemicarbazones show intense absorption of ultraviolet light (Heilbron, Johnson, Jones, and Spinks, 1942). The absorption spectra of the three compounds in chloroform were therefore examined by means of the Beckman photoelectric spectrophotometer (FIG. 1). They were quite suitable for the purpose of estimation provided that a method of extraction from biological fluids could be devised. It was found that 6057 and 8388 could be extracted by the same simple procedure but that 6082 could not. Descriptions of the methods and of their use in animal experiments follow.

1. *Estimation of* p-*anisaldehyde thiosemicarbazone* (6057) *and* p-*ethylsulphonylbenzaldehyde thiosemicarbazone* (8388) *in blood*

Reagents

1. 0.2*M*-disodium hydrogen phosphate, preserved by adding a trace of chloroform.
2. B.P. chloroform.
3. 0.2 g./100 ml. stock solution of 6057 or 8388 in methanol (stable in the dark).
4. 1 mg./100 ml. standard solution, prepared just before use by diluting the stock solution with water.

Procedure

Pipette 2 ml. of blood into 4 ml. of 0.2*M*-disodium hydrogen phosphate in a 60 ml. glass-stoppered bottle. Add 40 ml. of chloroform and shake for 5 min. An emulsion rarely forms, and can always be resolved by centrifuging the bottle. Withdraw most of the lower layer and clarify it by filtration through Whatman No. 1 or similar semi-fine paper, or by shaking it in a clean dry bottle with about half a gram of anhydrous sodium sulphate, A.R. Transfer it to a 10 cm. silica spectrophotometer-cell, and read the optical density at 325 mμ (6057) or 332 mμ (8388) against a blank. Let the reading be "*a*." Prepare the blank and a standard at the same time as the unknown by substituting 2 ml. of distilled water, and 2 ml. of the standard solution for blood in

the above procedure. Read the standard also against the blank. Let the reading be "*b*." Then the concentration of 6057 or 8388 in the unknown is *a*/*b* mg./100 ml. Normal blood gives a reading equivalent to about 0.05 mg./100 ml., which must be subtracted from the experimental results.

Notes

It was established that the graph of concentration against optical density was a straight line: a single standard was therefore adequate. Disodium hydrogen phosphate was added because previous experience with similar methods (Spinks, 1946) had shown that the extraction of interfering materials from blood was reduced when the blood was buffered to weak alkalinity. At first this caused difficulty because the disodium hydrogen phosphate used contained mercuric chloride as preservative. Extraction of 6057 was completely prevented, presumably because it formed a solvent-insoluble mercury salt. When a trace of chloroform was substituted for mercuric chloride recovery from water and blood was quantitative (Table I). The recovery from

TABLE I

RECOVERY OF 6057 AND 8388 FROM BLOOD (2 ML.)

μg. added		μg. found		% recovery	
6057	8388	6057	8388	6057	8388
2	5	1.83	4.96	91	99
4	10	3.97	9.25	99	92
6	15	5.88	15.1	98	101
10	25	9.50	25.0	95	100
14	35	13.3	34.3	95	98
20	50	20.0	46.2	100	92

plasma and urine was also quantitative. However, concentrations of 6057 in urine were later found to be very low, so that its presence had to be confirmed, and its amount assessed, by constructing a full absorption spectrum of each extract. Concentrations of 8388 in urine were much higher, and the method described could be used without modification.

2. *Estimation of* p-*hydroxybenzaldehyde thiosemicarbazone* (6082) *in plasma*

Reagents

1. 4*M*-sodium dihydrogen phosphate.
2. B.P. chloroform.
3. 1 g./100 ml. sodium carbonate.

4 and 5. Solutions of 6082 as described for 6057 and 8388.

Procedure

Pipette 2 ml. of plasma (which must not be contaminated by haemolysis) into a bottle. Add 2 ml. of 4*M*-sodium dihydrogen phosphate and 40 ml. of chloroform. Shake vigorously for 5 min. and filter the lower layer into a measuring cylinder. Transfer an aliquot of 30 ml. to a dry bottle. Add 8 ml. of 1 per cent sodium carbonate and shake vigorously for 3 min. Decant the upper layer into a test tube, centrifuge it for 5 min., and transfer it to a 2 cm. spectrophotometer cell. Read the optical density ("*a*") against a blank at 330 mμ. The blank and a standard are prepared by substituting 2 ml. of water and 2 ml. of a 1 mg./100 ml. standard solution of 6082 for plasma in the above procedure. If the reading of the standard is "*b*" the concentration of 6082 in the unknown is *Fa*/*b* mg./100 ml., where *F* is a recovery factor derived from data such as those of Table III.

Notes

Only a trace of 6082 was extracted from water or blood under the conditions used for 6057. This was shown to be due to two effects: first, 6082 was a stronger acid (*p*Ka about 9.5) than its phenolic structure had suggested (Table II); and second, it could not be fully extracted even at low *p*H values unless salted out by a high concentration of buffer (Table II). Further, when a *p*H of 4.8 and a high

TABLE II

EFFECT OF *p*H OF ADDED BUFFER, AND MOLARITY OF ADDED NaH_2PO_4 ON THE EXTRACTION OF 6082 FROM WATER INTO CHLOROFORM

*p*H of 0.2 *M*-buffer	% Extraction	Molarity of NaH_2PO_4 (*p*H 4.8)	% Extraction
5.0	48	4.0	101
6.0	45	2.0	88
7.0	46	1.0	70
8.0	41	0.5	60
8.9	32	0.2	52
10.6	6.5		
10% Na_2CO_3	0		

buffer concentration were used in the extraction of blood very large amounts of interfering material, equivalent to several mg. of 6082/100 ml., were extracted; and even then the recovery of 6082 was only about 20 per cent. Consequently, plasma was analysed instead. Interfering material was again removed, but in less amount, and when the 6082 was re-extracted into 1 per cent sodium carbonate (cf. Table III) the blank was reduced almost to zero. The recovery of 6082 from plasma was incomplete but adequate (Table III). The recovery from urine was theoretical; but 6082, like 6057, reaches only low concentrations in urine, and full absorption spectra of extracts must be constructed.

TABLE III

RECOVERY OF 6082 FROM PLASMA

Added, μg.	Found, μg.		% Recovery (Na_2CO_3)
	1st extraction ($CHCl_3$)	Re-extraction (1% Na_2CO_3)	
0	6.17	0.07	—
4	9.75	3.00	73
10	14.0	7.24	72
20	19.3	14.9	74
30	26.2	23.2	77
40	32.4	31.2	78
0	4.25	0	—
20	18.9	12.8	64
40	39.0	29.0	72
		Mean:	73

A disadvantage of analysing 6082 in plasma was that much data had already been obtained on the concentration of 6057 in blood. However, the validity of comparing blood with plasma concentrations in this series is indicated by the virtual identity of concentrations of 6057 in blood and plasma and by the fact that concentrations of 8388 in plasma are about 70 per cent of those in blood (results not shown). Another important point is that the major difference between the compounds is in rate of elimination from the blood; its apparent value could hardly be affected by analysing plasma instead of blood.

3. *Animal Experiments*

(*a*) *Mice.*—Each compound was given to groups of 4 mice in doses of 500 mg./kg., administered orally by syringe and blunt needle as a 2 per cent aqueous dispersion. Groups were killed at intervals after dosing by withdrawing heart blood under heavy chloroform anaesthesia. Blood from the 4 animals of a group was pooled for analysis. The concentrations found are shown in Fig. 2, which also includes the results of a similar experiment on 6057 in rats.

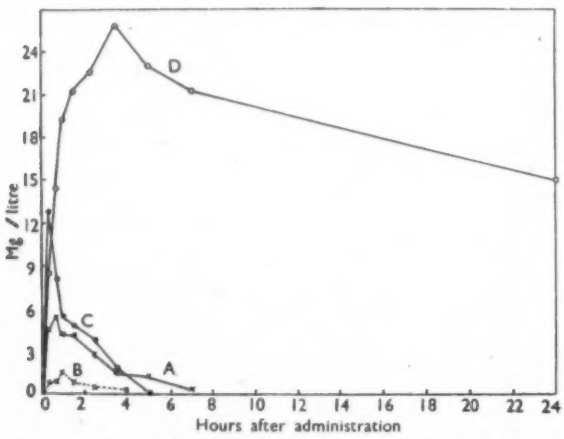


FIG. 2.—Concentrations of 6057 in mouse (A) and rat (B) blood, 6082 in mouse plasma (C) and 8388 (D) in mouse blood after the oral administration of 500 mg./kg. Ordinates: Milligrams per litre. Abscissae: Hours after administration.

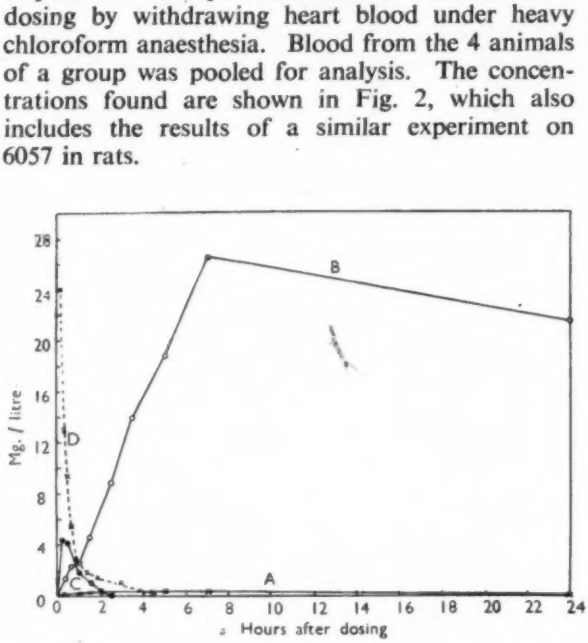


FIG. 3.—Concentrations of 6057 (A) and 8388 (B) in blood, and 6082 (C) in plasma, after oral doses of 250 mg./kg. in rabbits. Each graph is the average of three experiments. Concentrations of 6057 in blood (D) after i.v. administration of 40 mg./kg. to a single rabbit are also shown. Ordinates: Milligrams per litre. Abscissae: Hours after administration.

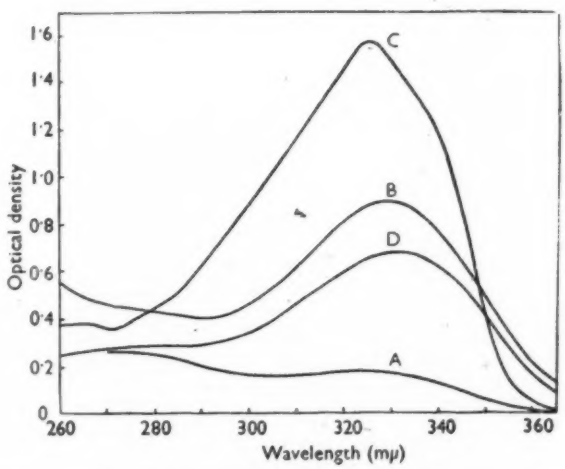


FIG. 4.—Absorption spectra of chloroform extracts of the first day's urine from rabbits receiving 6057 (A) and 8388 (B), and rats receiving 6057 (C), and of a 54 hour-blood sample (D) from a rabbit receiving 8388. Ordinates: Optical density. Abscissae: Wavelength (mμ).

(*b*) *Rabbits.*—Each compound was administered orally to rabbits as a 10 per cent aqueous dispersion, given by syringe and rubber catheter in doses of 250 mg./kg. 6057 was also administered intravenously as a 2 per cent dispersion in a dose of 40 mg./kg. Rabbits were bled from a marginal ear vein at intervals after dosing. The results are shown in Fig. 3. The identity of 8388 in a sample of rabbit blood taken 54 hours after dosing was confirmed spectrophotometrically (Fig. 4). 8388 could be detected in rabbit blood under these conditions for about a week.

Excretion of the three compounds in urine was examined using some of the above rabbits. Fig. 4 shows the absorption spectrum of an extract of the first day's urine of a rabbit receiving 6057. It was estimated from such curves that the amount of 6057 excreted in the urine did not exceed 1 per cent of that administered (Fig. 5); 6082 behaved similarly.

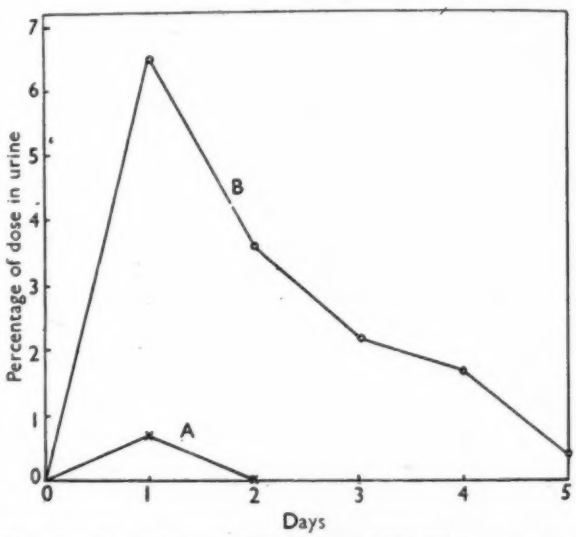


FIG. 5.—Excretion of 6057 (A) and 8388 (B) in the urine of typical individual rabbits after oral doses of 250 mg./kg. Ordinates: Percentage of dose in urine. Abscissae: Days.

Rats excreted about 2 per cent of an oral dose of 500 mg. of 6057/kg. Here the smaller urine volume permitted definite identification of the excreted material (Fig. 4). 8388 was readily identified even in rabbit urine (Fig. 4). Its behaviour was in sharp contrast to that of 6057 or 6082, since larger amounts appeared in the urine, and excretion continued over several days (Fig. 5). However, only about 10–14 per cent of the amounts administered to three rabbits were accounted for in the urine. Unfortunately, the analytical methods could not be applied to faeces, but it is improbable from analogy with other compounds of similar simplicity that much material was lost in the faeces. It may reasonably be assumed that most of each compound was degraded in the body (cf. Discussion).

DISCUSSION

The most important finding is that whereas 6057 and 6082 are rather rapidly eliminated, and attain only low blood or plasma concentrations, 8388 is very persistent and attains high blood concentrations. The marked difference between 8388 and the other two compounds is particularly evident when the areas beneath the blood concentration-time curves are measured. The figures for mice, expressed in arbitrary units, are: 6057, 2.4; 6082, 3.1; 8388 (to 72 hrs. after dosing) 154.

The results are in excellent agreement with the antituberculous properties of the three compounds, since 8388 is the least active *in vitro* (Martin, 1948) and the most active *in vivo* (Hoggarth *et al.*, 1949). It may reasonably be assumed that repeated administration during the therapeutic tests would lead to differences in blood concentration even more marked than those recorded here. The agreement between concentration and therapeutic effect suggests that the thiosemicarbazones act directly and not after conversion to metabolites.

It is important to consider the likely reasons for the difference between 8388 and 6057 or 6082, because of their possible bearing on the design of compounds that might attain blood concentrations similar to those of 8388 and have intrinsic activity comparable with that of 6057 or 6082. At present this consideration must necessarily be highly speculative. The nature of the blood concentration-time curves indicates that the main pharmacological difference between the compounds is in rate of elimination from the blood. Both 6057 and 6082 appear to be rapidly absorbed, probably more rapidly than 8388. Because most of each compound is degraded in the body it follows that the differences in the rates of elimination are mainly conferred by variation in the rate or manner of degradation. This is further supported by the fact that 8388, which is the most persistent compound, is nevertheless excreted in larger amount than the other two.

At first sight, the most likely point of chemical attack is the thiosemicarbazone group. If this be so it must be assumed that the group is readily attacked in 6057 and 6082, and only attacked with difficulty in 8388. Some weak indirect evidence in support of this assumption is available, in that Hoggarth (1948) has shown that a methoxy group in the *para* position of certain compounds related to benzaldehyde thiosemicarbazone renders the thiosemicarbazide

residue more susceptible to scission. However, a spectrophotometric search for metabolites in extracts of urine from rabbits receiving 6057 failed to indicate the presence of free or conjugated anisic acid, which is the most likely end-product of attack on the thiosemicarbazone group. Free or conjugated mercaptotriazole and thiodiazole also appeared to be absent. Both of these are potential cyclization products of thiosemicarbazones; the thiodiazole can be readily obtained *in vitro* by mild oxidation. The examination of the urine was not exhaustive, and other products may well have been present. In particular, *p*-hydroxybenzoic acid would probably not have been extracted by the solvents used.

A second possibility exists, since the methoxy and hydroxy *para* substituents of 6057 and 6082 are known to be capable of metabolic modification, whereas the ethylsulphonyl group of 8388 is thought not to be. For example, *p*-methylsulphonylacetanilide is a metabolic end-product of *p*-methylthioaniline in the mouse, rat, and rabbit (Rose and Spinks, 1948), and sensitive tests failed to detect sulphanilic acid derivatives in the urine of these animals. Similar results were obtained with the ethylsulphonyl homologue (Spinks, 1948). It is therefore probable that no further breakdown of the alkylsulphonyl group occurred. These considerations suggest that 6057 may be demethylated in the body to give 6082; 6082 would probably be conjugated with sulphuric acid or glucuronic acid. The conjugated derivatives when hydrolysed, would give *p*-hydroxybenzaldehyde which would almost certainly have escaped detection in the search for metabolites referred to above. These views do not directly explain the excretion of only a part of the 8388, but do so if the further assumption is made that the thiosemicarbazone group is slowly, and the *para* substituents of 6057 and 6082 are rapidly, attacked.

A third possibility is that each compound is hydroxylated in the body according to known metabolic reactions, and then conjugated: 6057 would give a guaiacol, and 6082 a catechol derivative; 8388 would probably be hydroxylated in the position *meta* with respect to the ethylsulphonyl group. It can be assumed that this *meta* hydroxylation would be less facile than the *ortho* hydroxylation of 6057 or 6082. Some support is available for the last two possibilities in that urine from rabbits receiving 6082 contains a water-soluble compound having an intense absorption spectrum with a sharp maximum at 315 mμ. The amount of this metabolite is estimated to be equivalent to about 60 per cent of the amount of 6082 administered, if similar intensities are assumed for the respective absorption spectra. This observation, made after the first draft of this paper had been written, shows, first, that 6082 is not excreted in the faeces to any marked extent, and, second, that the thiosemicarbazone group may well have remained intact, or may have been only partly degraded, say to the corresponding semicarbazone: simpler metabolites would be expected to absorb at shorter wavelengths. Otherwise there is little evidence in favour of any one of the theoretically possible methods of degradation. Indeed it is conceivable that all three could occur. However, they all suggest somewhat similar conclusions, i.e., that new antituberculous thiosemicarbazones should be sought among compounds having an unreactive *meta* directing group, or, less probably, an unreactive *ortho-para* directing group in the *para* position. It is possible that the requirements for high intrinsic activity and high blood concentrations may be incompatible; and the numerous examples of weakly active compounds belonging to one or other of these two suggested types (Hoggarth *et al.*, 1949) favour this view. It is hoped that a conclusion may be reached, first by studying the metabolism of the three compounds mentioned here, second by examining the blood concentrations of other compounds. A start has been made by determining the concentrations attained by *p*-acetamidobenzaldehyde thiosemicarbazone, which has been tested in man by Domagk's clinical collaborators (Moncorps and Kalkoff, 1947; Kuhlmann, 1948). So far it appears that this compound gives concentrations in mice similar to those of 6057 and 6082, and that it lacks the favourable persistence of 8388.

Summary

Spectrophotometric methods of estimating the thiosemicarbazones (6057, 6082, and 8388 respectively) of *p*-anisaldehyde, *p*-hydroxybenzaldehyde and *p*-ethylsulphonylbenzaldehyde have been devised.

6057 and 6082 are rapidly eliminated and attain only low blood or plasma concentrations in mouse and rabbit. 8388 is highly persistent and attains high blood concentrations. Only traces of 6057 and 6082 appear in rabbit urine. They are probably almost completely metabolized by the rabbit. 8388 is excreted unchanged in larger, but not theoretical, amounts.

The bearing of these results on the antituberculous activity of this group of compounds has been discussed.

The author is indebted to Dr. E. Hoggarth for helpful discussion, and to Mrs. A. Horton for skilled technical assistance.

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Note added in proof

The thiosemicarbazones were found to be photolabile during this work, but no evidence of photodecomposition was obtained during the estimations, which were carried out in winter. Repetition of some of them recently has shown that in summer decomposition by diffused daylight can occur, and estimations are now carried out in a darkroom, under diffused tungsten illumination. Readings of the standards indicate whether or not decomposition has occurred.

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THE EFFECT OF DITHIOLS ON SURVIVAL TIME IN RATS AND MICE POISONED WITH ORGANIC ARSENICALS

BY

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(Received May 19, 1949)

The usual method for observing the anti-arsenical activity of dithiols has been to inject two groups of animals with a lethal dose of the arsenical, to treat one group with a dithiol and to compare the mortalities in the two groups (Stocken and Thompson, 1946; *et al.*). As Box and Cullumbine (1947) have pointed out, the use of a quantal response (percentage mortality) for quantitative studies of this sort has considerable disadvantages: the dose range in which there is a suitable mortality is small; large numbers of animals must be used to give accurate estimates of mortality; the accuracy of the estimates depends on the observed mortalities and must be weighted accordingly; and it is difficult or impossible to introduce a number of factors into the experiment and to detect interactions between them. Box and Cullumbine suggested that similarly useful information might be obtained much more efficiently by using the times of survival after a lethal dose of poison instead of the mortalities. Weatherall (1945, 1949) observed that animals poisoned with oxophenarsine and treated with doses of dithiols insufficient to save life lived longer than control animals given no dithiol, and showed that in the controls the log survival time was roughly linearly related to the log dose of oxophenarsine. It was therefore decided to investigate the relation between dose of arsenical and survival time more fully, and to study the effect of dithiols on this relation, in order to see whether a more satisfactory method of measuring anti-arsenical activity could be so obtained.

Materials and Methods

Solutions of phenylarsenoxide were prepared by dissolving crystalline phenylarsenoxide (m.p. 159–161° C.) in 3*N* sodium hydroxide, neutralizing with 6*N* hydrochloric acid as nearly as possible without precipitation, and diluting with water until isotonic and then with 0.9 per cent (w/v) sodium chloride solution. Solutions of oxophenarsine (mapharside) were prepared in distilled water from pure material kindly provided by Messrs. Parke Davis and Co. The dithiols used are described in Table I. The preparation and chemical properties have been or will be described elsewhere (Evans and Owen, 1949; Evans, Fraser, and Owen, 1949). Solutions of 1 : 2-dimercaptopentane-3 : 4 : 5-triol, 1 : 2-dimercaptohexane-3 : 4 : 5 : 6-tetrol, dimercaprol glucoside (BAL-Intrav) and 3(2′ : 3′-dimercaptopropyl)-mannitol were prepared by dissolving their barium salts in water, adjusting the *p*H to 6 with 10*N* sulphuric acid, removing any remaining barium ions with saturated sodium sulphate, and removing the barium sulphate by centrifuging for 15 min. at 2,500 r.p.m. The complete removal of barium ions was checked by adding a further trace of saturated sodium sulphate and observing the absence of a precipitate. The concentrations of these solutions were estimated at the time of experiments as follows: sufficient hydrochloric acid was added to make the solutions of normal acidity, and aliquots were then titrated at 0° C. against *N*/10 or *N*/50 iodine in potassium iodide with a few drops of 1 per cent (w/v) starch solution as indicator until a blue colour persisted for 30 sec. 2 : 3-Dimercaptopropionic acid was dissolved in a minimal amount of 3*N* sodium hydroxide before dilution with 0.9 per cent sodium chloride. Solutions of other dithiols in 0.9 per cent sodium chloride or in olive oil were diluted so that the volume of each injection was 5.0 ml./kg. All doses have been expressed in microgramme-molecules (μM) per kg. body weight, in order to facilitate the comparison of chemically equivalent quantities of different substances, and in order to avoid doses numerically smaller than unity and therefore having negative logarithms.

Young female albino rats weighing 55.9±11.9 (S.D.) grammes and adult male mice weighing 20.2±2.9 (S.D.) grammes were used. Experiments were done in a thermostatic room; the temperature varied between 21° C. and 25° C. on different days, but it was checked half-hourly during experiments and rarely varied by more than ±0.5° C. during each experiment. Food was withheld from the animals on the evening before an experiment, and on the following morning each animal was put in a numbered glass jar with a wire mesh top. They were then selected for different treatments by use of a table of random numbers (Fisher and Yates, 1943, p. 90). Each animal was injected intramuscularly with an arsenical in one limb and immediately afterwards with a dithiol or saline in the opposite limb. The time of injection was recorded. For practical convenience the animals in a group receiving the same treatment were

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TABLE I

Substance	Formula	Mol. wt.	Solvent used	LD50 μM/kg.	Species	Route of administration	Reference
2 : 3-dimercaptopropanol (Dimercaprol, BAL)	$CH_2SH.CHSH.CH_2OH$	124	0.9% NaCl	1,220	Rat	Intramuscular	McDonald (1948)
				910	,,	,,	Stocken and Thompson (1946)
				850	,,	,,	Fitzhugh *et al.* (1946)
				1,000	Mouse	,,	Weatherall (1949)
1 : 2-dimercaptobutane-3 : 4-diol (1 : 2-dithiothreitol)	$CH_2SH.CHSH.CHOH.CH_2OH$	154	,,	1,000	,,	,,	Weatherall (1949)
1 : 2-dimercaptopentane-3 : 4 : 5-triol	$CH_2SH.CHSH.(CHOH)_2CH_2OH$	184	,,	1,700–2,500	,,	,,	,, ,,
1 : 2-dimercaptohexane-3 : 4 : 5 : 6-tetrol	$CH_2SH.CHSH.(CHOH)_3CH_2OH$	214	,,	1,700	,,	,,	,, ,,
Dimercaprol glucoside (BAL-Intrav)	$CH_2SH.CHSH.CH_2OC_6H_{11}O_5$	286	,,	3,800–4,700	,,	,,	,, ,,
2 : 3 : 4 : 6-tetramethyl-2′ : 3′-dimercaptopropyl glucoside	$CH_2SH.CHSH.CH_2OC_6H_7O(OCH_3)_4$	342	Olive oil	5,000–8,000	,,	,,	,, ,,
3(2′ : 3′-dimercaptopropyl) mannitol	$CH_2SH.CHSH.CH_2OCH$ with $(CHOH)_2CH_2OH$ and $CHOH.CH_2OH$	288	0.9% NaCl	3,200	,,	,,	,, ,,
1 : 2-dimercaptopropionic acid	$CH_2SH.CHSH.COOH$	138	NaOH and 0.9% NaCl	600	Rat	,,	Fitzhugh *et al.* (1946)
δ-mercapto-γ-valerothiolactone	$CH_2SH.CH.CH_2CH_2CO$ with S bridge	148	0.9% NaCl	900	Mouse	,,	Weatherall (1949)
1 : 6-hexanedithiol	$CH_2SH.(CH_2)_4CH_2SH$	150	,,	ca.700	Cat	Intravenous	Chenoweth *et al.* (1946)
3-acetoxy-1 : 2-bisacetylthiopropane (triacetyl dimercaprol)	$CH_2(S.CO.CH_3)CH(S.CO.CH_3)CH_2O.CO.CH_3$	250	Olive oil	1,600	Mouse	Intramuscular	Weatherall (1949)

injected consecutively, and the groups in which the largest and smallest survival times were expected were injected first and last respectively. As is indicated later, this concession to practical convenience was perhaps undesirable. The animals were observed about every two minutes for the first few hours and later at longer intervals for forty-eight hours. An animal was deemed dead when it made no respiratory movement while watched for thirty seconds. The time of death was taken as the mean of the time when the animal was last observed alive and the time when it was first observed dead. When the logarithmic or reciprocal transformation of the survival times was used, the mean of the logarithms or reciprocals of the times last seen alive and first seen dead was taken. In order to avoid confusion with means derived from groups of animals, the values for individual animals obtained as just described are referred to hereafter as the survival time, log survival time, and reciprocal survival time, and the word mean is used only in connexion with groups of animals. Any animal which survived longer than forty-eight hours from the time of injection was killed and not counted in the estimation of the mean survival time of its group, except when the reciprocal transformation was used. In fact, only 6 out of 678 animals survived the experimental period. Food and water were withheld during the first ten hours after injection and were then provided *ad libitum*, though the animals seldom showed any desire to eat or drink.

The statistical procedures and symbols used follow the practice of Fisher (1944), unless it is otherwise indicated.

TABLE II

THE EFFECT OF DIMERCAPROL ON THE MEAN LOG SURVIVAL TIME OF PHENYLARSENOXIDE POISONED RATS

Exp.	Dose of dimercaprol		Log survival time in hours (Means and standard errors for groups of four rats)				Mean increment in log survival time† Log hours
			Dose of phenylarsenoxide				
	μM/kg.	Log μM/kg.	μM/kg. 80 Log μM/kg. 1.90	113 2.05	160 2.20	226 2.35	
A	Nil		+0.063±0.0436	+0.003±0.0824	−0.047±0.0515	−0.339±0.0593	—
B			+0.120±0.0738	−0.229±0.0855	−0.380±0.1358	−0.606±0.0305	—
C			+0.094±0.0974	+0.026±0.1713	−0.200±0.0725	−0.519±0.0691	—
D			+0.068±0.0681	−0.262±0.0504	−0.477±0.0661	−0.698±0.0391	—
D	16	1.20	+0.035±0.0336	−0.199±0.0362	−0.437±0.0196	−0.690±0.0293	0.020±0.3555
D	32	1.50	+0.015±0.0148	−0.081±0.0412	−0.394±0.0418	−0.664±0.0642	0.061±0.0372
D	46	1.66	+0.137±0.0570	−0.240±0.2132	+0.048±0.1114	−0.680±0.0380	0.159±0.0687
C	80	1.90	+0.538±0.0940	+0.295±0.0524	+0.229±0.0497	−0.225±0.0735	0.359±0.0653
A	113	2.05	+0.943±0.1292	—	—	—	—
B			+0.676±0.1228	—	—	—	—
C			+0.618±0.0736	+0.506±0.0490	+0.447±0.0756	+0.001±0.0533	0.544±0.0638
A	160	2.20	+1.047±0.0696	+0.832±0.0799	—	—	0.907±0.0499
B			+1.104±0.0528	+0.697±0.0481	—	—	0.955±0.0472
C			+0.964±0.0571	+0.608±0.0351	+0.560±0.0788	+0.135±0.0597	0.717±0.0628
D			+1.027±0.1626	—	+0.410±0.0681	+0.029±0.0918	0.858±0.0645
A	226	2.35	+1.114±0.0340*	+0.914±0.0247	+0.742±0.0542	+0.317±0.0704	0.852±0.0391
B			—	+1.171±0.1091	+0.513±0.0294	+0.327±0.0496	1.072±0.0591
C			—	+0.728±0.0862	+0.727±0.0474	+0.293±0.0366	0.814±0.0648
A	320	2.50	—	+1.015±0.0468	+1.015±0.1156	+0.473±0.0222	0.962±0.0492
B			—	+1.378±0.0013*	+0.770±0.0829	+0.571±0.0614	1.311±0.0567
C			—	—	+0.977±0.0343	+0.494±0.0347	1.095±0.0394
A	452	2.65	—	—	+1.060±0.0529	+0.889±0.0463	1.168±0.0372
B			—	—	+1.081±0.1298	+0.880±0.0822	1.474±0.0733
C			—	—	—	+0.654±0.0959	—

* One survivor in group. † Calculated as described in text.

RESULTS

In general it was found both for oxophe arsine and for phenylarsenoxide that the survival time became shorter as the dose increased, and that for a given dose of poison the survival time of animals treated with dimercaprol became longer as the dose of dimercaprol was increased. The relations were studied first with oxophenarsine, but, as clearer results were obtained with phenylarsenoxide, the latter will be presented first and the data for oxophenarsine thereafter summarized briefly, mainly to indicate the differences observed.

I. Survival time of rats poisoned with phenylarsenoxide with or without treatment with dimercaprol.—No information has been found about the LD50 of phenylarsenoxide in rats, but it is reported to be about 12 μM/kg. intraperitoneally in mice (Eagle, Doak, Hogan, and Steinman, 1940). In the present experiments intramuscular doses of 36 μM/kg. and over killed all rats not treated with dithiols, and doses of 80 μM/kg. and over were nearly always fatal when the amount of dithiol did not exceed twice the equivalent amount of phenylarsenoxide. As survival was not desired, doses of the latter order were used, with the results shown in Table II and Fig. 1.

Sets of sixteen rats were divided into four groups of four, and the groups were injected with 80, 113, 160, and 226 μM/kg. respectively of phenylarsenoxide. Some sets received a dose of dimercaprol which was constant for the set, and one set, the controls, were treated with 0.9 per cent sodium choride solution. Particularly in early experiments the dimercaprol-treated sets were sometimes incomplete, either because groups in which survivors were expected were omitted or because the ranges being explored required more rats than were available or manageable to fill all the groups included in the range. When the mean survival times of the groups in each set were plotted against the dose or the log dose of phenylarsenoxide the points lay on a curve and the variance increased as the mean survival time increased. Two transformations of the data were therefore examined. When the means of the reciprocals of the survival times (cf. Box and Cullumbine, 1947) were plotted against the dose or the log dose of phenylarsenoxide, the points again lay on a curve and the variance still increased as the means increased. But when the means of the logarithms of the survival times were plotted against the log doses of phenylarsenoxide, the points lay roughly on a straight line and the variances showed only slight positive correlation with the means. Moreover, the lines for sets which received a constant dose of dithiol were approximately parallel to those for the set which received no dithiol in the same experiment; and there was no obvious progressive change of slope with increasing doses of dimercaprol. In order to assess the significance of the departures from linearity, analyses of variance were performed on those parts of the data which were symmetrically arranged (Table III). The results of these analyses were not altogether satisfactory. The significance of the regression of log survival time on log dose of phenylarsenoxide and the significance of the effect of dimercaprol, except in the smallest doses (experiment *D*), were indeed beyond question. But in experiments *A* and *C* the mean squared deviations from linearity were very significantly greater than the mean square attributable to random fluctuation, and experiments *B* and *D* showed a less marked but similar tendency. In experiment *B* there was also a greater departure from parallelism between the lines in the presence and absence of dimercaprol than would be expected by chance.

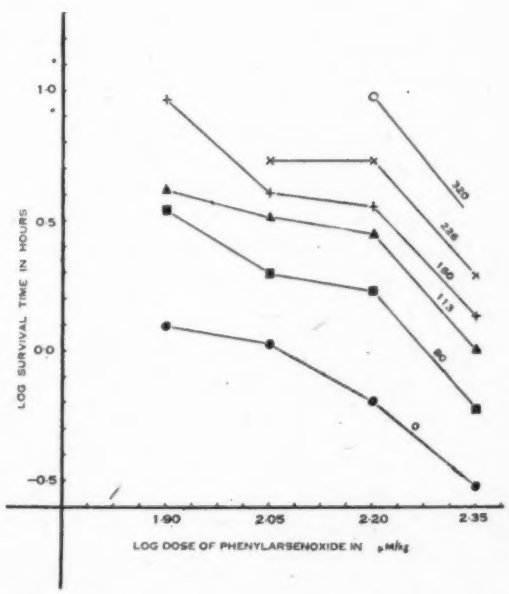

FIG. 1.—The log survival time of rats poisoned with phenylarsenoxide and treated with dimercaprol (experiment *C*). Ordinates: log survival time in hours. Abscissae: log dose of phenylarsenoxide, in μM/kg. ●———● Rats treated with 0.9 per cent sodium chloride. ■———■ Rats treated with dimercaprol, 80 μM/kg. ▲———▲ Rats treated with dimercaprol, 113 μM/kg. +———+ Rats treated with dimercaprol, 160 μM/kg. ×———× Rats treated with dimercaprol, 226 μM/kg. o———o Rats treated with dimercaprol, 320 μM/kg. All points indicate the mean for groups of four rats.

TABLE III

ANALYSIS OF VARIANCE OF THE LOG SURVIVAL TIMES OF RATS POISONED WITH PHENYLARSENOXIDE AND TREATED WITH DITHIOLS

The data analysed here are summarized in Tables II and VI. The values selected are, in experiments A and B, phenylarsenoxide 113, 160, and 226 μM/kg., dimercaprol 0, 226, and 320 μM/kg.; in experiments C, D, E, and F, phenylarsenoxide 80, 113, 160, and 226 μM/kg. and all doses of dithiols for which information was available at the same four doses.

The figures in parentheses are the number of degrees of freedom with which the means are estimated. The mean squares for linear regression and (except in experiment D) for treatment with dithiols are all significantly ($P<0.001$) greater than the mean square for random fluctuation. Other mean squares are printed in italics when they exceed the mean square for random fluctuation by a ratio greater than the 5 per cent point of e^{2s} and in bold type when the ratio is greater than the 1 per cent point.

Variance due to	Mean squares					
	Experiments with dimercaprol				Experiments with other dithiols	
	A	*B*	*C*	*D*	*E*	*F*
Linear regression on log dose of phenylarsenoxide	1.462(1)	2.742(1)	3.929(1)	4.346(1)	6.329(1)	8.534(1)
Deviations from linear regression	**0.239(1)**	*0.143(1)*	**0.159(2)**	*0.078(2)*	0.059(2)	0.037(2)
Treatment with dithiols	3.148(2)	5.855(2)	1.505(3)	0.076(3)	1.593(4)	2.896(4)
Differences of linear regression	0.036(2)	**0.135(2)**	0.016(3)	0.004(3)	*0.102(4)*	**0.199(4)**
Other interaction	0.019(2)	0.061(2)	0.017(6)	**0.099(6)**	0.023(8)	0.025(8)
Random fluctuation	0.017(27)	0.024(27)	0.025(48)	0.022(48)	0.034(60)	0.034(60)

If a sufficiently wide range of doses is examined, the regression of log survival time on log dose of phenylarsenoxide will not be linear. At the lower end of the scale survivals will occur and the log survival time will be practically infinite; and at the upper end a limit is likely to occur depending on the time necessary for the transport of phenylarsenoxide from the site of injection to the site of action and possibly for the accumulation there of toxic substances whose metabolism has been inhibited. If the departures from linearity showed a consistent tendency towards curvature convex towards the zero ordinate, it would not be surprising. But this is not the case: the observed irregularities appear to be distributed more or less fortuitously.

There appears to be a more plausible explanation of the irregularities. As was indicated above, animals in a group receiving the same treatment were injected consecutively, for reasons of practical convenience. The estimate of the random fluctuation, or error, of the experiment is based on the deviations occurring within the individual groups, and therefore does not include any measure of the variation in sensitivity of the animals occurring during the two or three hours in which the injections were performed. If there is any reason why such variation might be appreciable, the estimate of error cannot be regarded as satisfactory and tests of significance based on it will tend to underestimate the probability of observed discrepancies being due to chance. In fact there is present at least one detectable factor which may account for a greater variation between groups treated at different times than is observed within the groups. It was found that the slopes of the regression lines relating log survival time to log dose (in rats treated only with phenylarsenoxide) did not vary greatly from day to day. But the position of the line varied, and it appeared that this was due largely to the temperature of the room, and possibly to the weight of the rats. A multiple regression relating log survival time in hours (Y) to log dose of phenylarsenoxide in μM/kg. (d_P), body weight in grammes (w) and temperature of the room in °C. (t) was therefore calculated for 129 rats. The log survival time was found to be expressed by

$$Y = 5.46 - 1.57d_P - 0.002w - 0.098t$$

The regression for body weight was not significant ($0.2>P>0.1$), but those for dose and for temperature were highly significant ($P<0.001$ in each case). It therefore appeared that apart from the influence of dose, changes of temperature of as little as half a degree altered the log survival time by approximately 0.05 log hours, or 12 per cent. The temperature of the room in which the experiments were performed was not controlled more accurately than this, and the variation due to changes of temperature within these limits is clearly important. It therefore appears legitimate to regard the estimate of random fluctuation as too small and the significance of departures from linearity and parallelism as consequently overestimated.

No very good substitute is available, and probably the least unsatisfactory estimate of overall variation would be given by the mean of the sums of squares attributable to deviations from linear regression, to differences of linear regression, and to other interaction. Even if the largest mean square among these is taken as a basis of comparison, the regression itself and the effect of dimercaprol in sufficient doses are still most significant. Preferable estimates of the error of certain important comparisons are suggested below.

If the lines are parallel, a given dose of dimercaprol produces a constant increment in log survival time irrespective of the dose of phenylarsenoxide. On the assumption that apparent differences in slope were not appreciably more than could be attributed to chance, this increment was estimated by deducting the mean log survival time of a set of rats treated with various doses of phenylarsenoxide from the mean log survival time of a comparable set treated with the same doses of phenylarsenoxide and a constant dose of dimercaprol. (This is equivalent to estimating the mean of the increments produced by dimercaprol at each dose of phenylarsenoxide.) If Z symbolizes the mean increment in log survival time, $y_1, y_2, \ldots y_m$, the log survival times of individual animals treated with doses 1, 2, . . . m, of poison, and $z_1, z_2, \ldots z_m$, the log survival times of individual animals treated with the same doses of poison and a constant dose of antidote, and there are n animals in each group, then

$$Z = \frac{S(z_1) - S(y_1) + S(z_2) - S(y_2) + \ldots + S(z_m) - S(y_m)}{mn}$$

As the mean square for error given by the analysis of variance is likely to be too small, the variance of Z has been estimated from the deviations within the $2m$ groups concerned. If v_z is the variance of Z, then

$$V_z = \frac{S(z_1 - \bar{z}_1)^2 + S(y_1 - \bar{y}_1)^2 + \ldots + S(z_m - \bar{z}_m)^2 + S(y_m - \bar{y}_m)^2}{2m(n-1)}\left(\frac{1}{mn} + \frac{1}{mn}\right)$$

Values for Z and $\sqrt{V_z}$ are given in the last column of Table II.

The smallest doses of dimercaprol used had no significant effect on the survival time of phenylarsenoxide poisoned rats, and it appeared that about 40 μM/kg. was the threshold dose for this effect; 46 μM/kg. produced an appreciable increase in survival time, but this was not significant with the size of group used. With larger doses the relation between the mean increment in log survival time and the log dose of dimercaprol was practically linear, even when points obtained on different days were considered together (Fig. 2). A straight line was therefore fitted to all points where the log survival time was significantly increased (i.e., those obtained with doses of dimercaprol of 80 μM/kg. and over), as shown in Fig. 2. If Z is the mean increment in log survival time for a dose d_A of dimercaprol, this line has the formula

$$Z = 1.18d_A - 1.81$$

when Z is measured in log hours and d_A in log μM/kg.

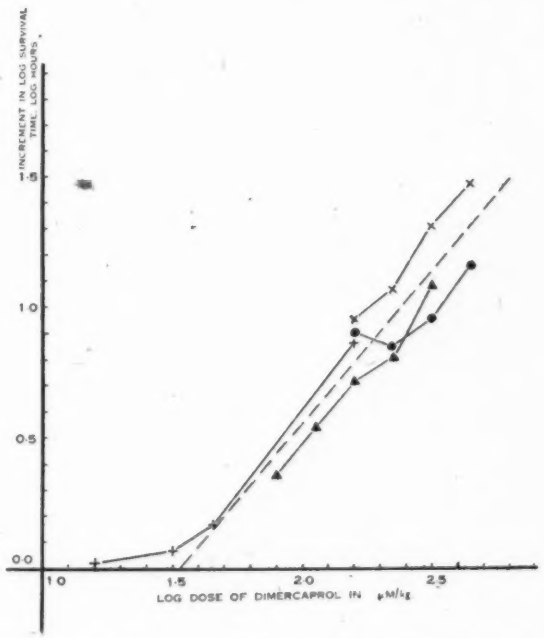

FIG. 2.—The increment in log survival time produced by dimercaprol in rats poisoned with phenylarsenoxide. Ordinates: mean increment in log survival time. Abscissae: log dose of dimercaprol in μM/kg. ●———● Experiment *A*. ×———× Experiment *B*. ▲———▲ Experiment *C*. +———+ Experiment *D*. The line of best fit (– – –) has been calculated from the equation $Z = 1.18d_A - 1.81$.

Examination of the data suggested that within the small range used in the present experiments, environmental temperature had no striking effect on the increment in survival time produced by dimercaprol, and as the data did not give systematic information on this point it was not pursued further.

A way of expressing these findings consists in plotting the dose of phenylarsenoxide against the dose of dimercaprol and drawing lines connecting points where the mean survival time is the same. Such lines may be called isochrons. In order to prepare these lines, the equations given above for the log survival time in the absence of dimercaprol and for the increment produced by dimercaprol have been used. In the first equation, the mean values for

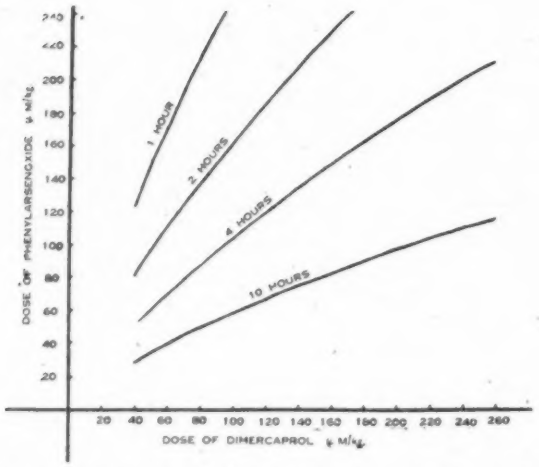

FIG. 3.—Isochronic lines for rats poisoned with phenylarsenoxide and treated with dimercaprol. Ordinates: dose of phenylarsenoxide in μM/kg. Abscissae: dose of dimercaprol in μM/kg. The values for the points have been calculated as described in the text. The lines, from top left to bottom right, connect points for survival times of 1, 2, 4, and 10 hours respectively.

w (56 g.) and t (22°) used in calculating the regression have been substituted, giving the relation between log dose and log survival time as

$$Y = 3.2 - 1.57d_P$$

Isochrons obtained by substitution for d_P and d_A are shown in Fig. 3. It will be seen that, within the present experimental range the isochronic lines are surprisingly nearly straight, even when the doses and not the log doses are plotted against each other. From these lines, or directly from the regression equations, the amount of phenylarsenoxide neutralized by a given dose of dimercaprol can be calculated. This quantity is not constant. Values for it derived from the one hour, four hour and ten hour isochrons at selected dimercaprol dose levels are shown in Table VII. Their significance is discussed later.

II. Survival time of rats and mice poisoned with oxophenarsine, with or without treatment with dimercaprol.—The LD50 of oxophenarsine given by intramuscular injection is reported to be in rats between 85 and 93 μM/kg. (Gruhzit, 1935) and in mice about 150 μM/kg. (Weatherall, 1949). Data on the relation of dose to survival time are presented in Tables IV and V. In both rats and mice the transformation log dose/log survival time gave an approximately linear relation with insignificant positive correlation ($r = +0.51$, $0.1 > P > 0.05$) between the mean and the variance, and with a slope varying between 1.7 ± 0.33 and 2.3 ± 0.43 on different days. Weatherall (1949), with much less accurate estimates of the survival time and with doses which only just caused 100 per cent mortality, found a slope of 2.76 in mice. This confirms the expectation that the line is actually convex downwards and can be regarded as approximately straight only over a limited range. The experiments with dimercaprol were designed at a time when it was expected that the dose of dithiol necessary to produce a constant increment in log survival time would be proportional to the dose of poison used. Consequently data are not available for the effect of the same absolute dose of dimercaprol with different doses of oxophenarsine, and lines comparable to those described above for phenylarsenoxide cannot be fitted. The increments in log survival time produced by different doses of dimercaprol were not inconsistent with the hypothesis of a linear relation between the mean increment in log survival time

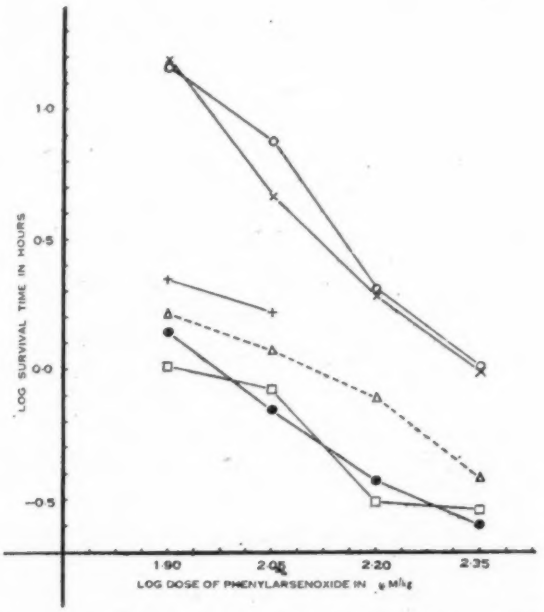

FIG. 4.—The log survival time of rats poisoned with phenylarsenoxide and treated with various dithiols (experiment *F*). Ordinates: log survival time in hours. Abscissae: log dose of phenylarsenoxide in μM/kg. ●———● Rats treated with 0.9 per cent sodium chloride. o———o Rats treated with dimercaprol, 160 μM/kg. ×———× Rats treated with 1:2-dimercaptobutane-3:4-diol. +———+ Rats treated with 1:2-dimercaptopentane-3:4:5-triol. □———□ Rats treated with 1:2-dimercaptohexane 3:4:5:6-tetrol. △- - - - -△ Rats treated with δ-dimercapto-γ-valerothiolactone. All points indicate the mean for groups of four rats.

TABLE IV

THE EFFECT OF DIMERCAPROL ON THE LOG SURVIVAL TIME OF OXOPHENARSINE POISONED MICE

Exp.	Dose of dimercaprol		Log survival time (Means and standard errors for groups of ten mice)				
			Dose of oxophenarsine				
	μM/kg.	Log μM/kg.	μM/kg. 250 Log μM/kg. 2.40	320 2.50	400 2.60	500 2.70	600 2.78
a	Nil	—	+0.712±0.0950	+0.391±0.0472	+0.284±0.0576	+0.064±0.0199	—
b			—	+0.357±0.0442	+0.457±0.0824	—	−0.277±0.1039
c			—	+0.437±0.0371	—	—	—
d			—	+0.516±0.0256	—	+0.176±0.0478	—
e			—	+0.460±0.0337	—	+0.029±0.0557	—
c	80	1.90	—	+0.317±0.0390	—	—	—
d			—	+0.477±0.0479	—	—	—
e			—	+0.466±0.0229	—	—	—
d	125	2.10	—	—	—	+0.382±0.0266	—
e			—	—	—	+0.324±0.0219	—
c	160	2.20	—	+0.593±0.0101	—	—	—
d			—	+0.608±0.1152	—	—	—
e			—	+0.424±0.0391	—	—	—
d	250	2.40	—	—	—	+0.388±0.1223	—
e			—	—	—	+0.374±0.0285	—
a	320	2.50	—	+0.814±0.1721†	—	—	—
b			—	+0.650±0.1203*	—	—	—
e			—	+0.774±0.1297*	—	—	—
a	400	2.60	—	—	+0.639±0.0781	—	—
b			—	—	+0.797±0.1772	—	—
a	500	2.70	—	—	—	+0.458±0.0701	—
e			—	—	—	+0.372±0.0504	—

* One survivor in group. † Two survivors in group.

TABLE V

THE EFFECT OF DIMERCAPROL ON THE LOG SURVIVAL TIME OF OXOPHENARSINE POISONED RATS

Dose of dimercaprol		Log survival time in hours (Means and standard errors for groups of ten rats)		
		Dose of oxophenarsine		
μM/kg.	Log μM/kg.	μM/kg. 320 Log μM/kg. 2.50	400 2.60	500 2.70
Nil	—	+0.011±0.0268	−0.072±0.0561	−0.282±0.0689
80	1.90	+0.190±0.0408	—	—
100	2.00	—	+0.028±0.0656	—
125	2.10	—	—	+0.021±0.0546
200	2.30	—	+0.149±0.0746	—
320	2.50	+0.589±0.1263	—	—
400	2.60	—	+0.404±0.1012	—
500	2.70	—	—	+0.647±0.1009

and the log dose of dimercaprol. The slope, however, appeared to be substantially less than with phenylarsenoxide (0.5 for oxophenarsine in mice, 0.6 for oxophenarsine in rats, and 2.2 for phenylarsenoxide in rats) and the threshold dose of dimercaprol was higher (about 80 μM/kg.). Below this dose, at least in mice, actual acceleration of death was observed, although only once (Table IV, exp. *c*) was this large enough to be significant with the size of group used.

III. Survival time of rats poisoned with phenylarsenoxide and treated with other dithiols.—As in the experiments with dimercaprol, sets of sixteen rats were divided into four groups of four and phenylarsenoxide was injected as before. One set received also an intramuscular injection of 0.9 per cent sodium chloride solution, one set the largest dose of dimercaprol which was expected not to save any lives (160 μM/kg.), and the remaining sets the same dose of other dithiols (Table VI and Fig. 4). This dose was in only one case greater than one-quarter of the LD50 of the dithiol (Table I). In certain instances, titration at the end of the experiment indicated that the solutions contained less dithiol than expected. In these cases, the dose was calculated from the results of the titration. Insufficient material was available to complete the lines for certain dithiols. The variance of the symmetrically arranged data was analysed as before (Table III). For no obvious reason, in neither experiments *E* nor *F* were the departures from linear regression of log survival time on log dose of phenylarsenoxide significantly greater than the mean square for error, although the initial fault in the experimental design had not been amended. This suggests that whatever in the dimercaprol experiments increased the variation between groups, whether temperature changes or some other unsuspected factor, was less actively operative; and due weight must therefore be attached to the differences of linear regression, which are significantly greater than can be accounted for by random fluctuation. The lack of parallelism is hardly surprising. Different thiols are likely to be absorbed and excreted at different rates. They will reach a peak concentration in the blood and tissues at different times, and so, for example, a slowly absorbed substance might show no appreciable activity against 226 μM/kg. of phenylarsenoxide, which kills rats in about ten minutes, and yet be quite active against 80 μM/kg. which is lethal in about an hour. Indeed, it can only be fortuitous that dimercaprol happened to be equally active, or nearly so, against all the doses of phenylarsenoxide used. Once lack of parallelism occurs, any estimate of activity based on the increment in survival time becomes an arbitrary measurement depending on the doses of phenylarsenoxide chosen. There is no obvious way of overcoming this difficulty. As the conditions in these experiments have been standardized, with four fixed doses of phenylarsenoxide and one dose of dithiol as nearly constant as was practicable, and as the lack of parallelism is not very great, the mean increments in log survival time still give an approximate indication of the activity of the thiol under these experimental conditions, and they have been calculated as before. Small differences in the mean increments, particularly when there are also differences in slope, are clearly unimportant, but in fact there were considerable differences in the ability of dithiols to increase the survival time. The significance of differences has been estimated as before, using the mean squared deviations of the groups concerned as the basis of comparison in a *t* test. No dithiol was more active than dimercaprol, and all except 1 : 2-dimercaptobutane-3 : 4-diol were significantly less active. Two substances (1 : 2-dimercaptohexane-3 : 4 : 5 : 6-tetrol and 1 : 6-hexanedithiol) accelerated death, the latter significantly. The other substances were intermediate in activity.

To facilitate quantitative comparisons, the activities have been estimated as percentages of the activity of dimercaprol, by assessing from the previously determined dose-response curve how much dimercaprol would have been necessary to produce the observed increments in log survival time. The pooled estimate of the slope of the dose response curve ($b = 1.18$) was used, and the position of the line was fixed by the observed mean increment in log survival time produced by the standard dose of dimercaprol. For example, in experiment *E*, 160 μM/kg. of dimercaprol increased the log survival time by 0.827 log hours, and substituting,

$$0.827 = 1.18 \times 2.20 + a,$$

whence $$a = -1.769$$

and the expected mean increment in log survival time, Z, for any log dose of dimercaprol, d_A, was given by $$Z = 1.18d_A - 1.769$$

By substituting for Z the mean increment in log survival time obtained for an unknown dithiol, e.g., 0.445 log hours for dimercaprol glucoside, the amount of dimercaprol to which the dose used of unknown dithiol was equivalent could be calculated.

Thus $$0.445 = 1.18d_A - 1.769$$

whence $$d_A = 1.875.$$

As the log dose of dimercaprol glucoside in this experiment was 2.146 μM/kg., its activity expressed as a ratio to that of dimercaprol was antilog (1.875–2.146) or 53.7 per cent. Values calculated similarly for other dithiols are shown in the third column from the right of Table VI.

TABLE VI

THE EFFECT OF VARIOUS DITHIOLS AND RELATED SUBSTANCES ON THE LOG SURVIVAL TIME OF PHENYLARSENOXIDE POISONED RATS

Exp.	Dose of dithiol			Log survival time in hours (Means and standard errors for groups of four rats)				Mean increment in log survival time. Log hours	Slope of line relating log dose of phenylarsenoxide to log survival time	Molar antiarsenical activity as a percentage of that of dimercaprol	Molar toxicity as a percentage of that of dimercaprol	Ratio of antiarsenical activity and toxicity
				Dose of phenylarsenoxide								
		μM/kg.	Log μM/kg.	μM/kg. 80 Log μM/kg. 1.90	113 2.05	160 2.20	226 2.35					
E	Nil	—	—	−0.010±0.0291	−0.188±0.0754	−0.449±0.0670	−0.491±0.0894	—	−1.15	—	—	—
	Dimercaprol	160	2.20	+1.171±0.0626	+0.583±0.1297	+0.327±0.0243	+0.190±0.0524	0.827±0.0519	−2.04	100	100	1.00
	Dimercaprol-glucoside	140	2.15	+0.732±0.0993	+0.300±0.1115	+0.012±0.0508	−0.302±0.1046	0.445±0.0585	−2.26	54	24	2.25
	3(2′: 3′-dimercaptopropyl) mannitol	140	2.15	+0.511±0.0695	+0.165±0.1046	+0.083±0.0530	−0.244±0.0479	0.388±0.0499	−1.56	48	31	1.53
	2: 3: 4: 6-tetramethyl-2′: 3′dimercaptopropyl glucoside	160	2.20	+0.232±0.0460	−0.006±0.1237	−0.187±0.1713	−0.374±0.1273	0.176±0.0716	−1.33	28	15	1.82
F	Nil	—	—	+0.146±0.0880	−0.156±0.0548	−0.434±0.0853	−0.596±0.0405	—	−1.67	—	—	—
	Dimercaprol	160	2.20	+1.161±0.1012	+0.875±0.0495	+0.308±0.0354	+0.010±0.0105	0.848±0.0477	−2.68	100	100	1.00
	1: 2-dimercaptobutane-3: 4-diol	160	2.20	+1.187±0.0568	+0.660±0.0513	+0.277±0.0407	−0.008±0.0401	0.789±0.0424	−2.64	89	100	0.89
	1: 2-dimercaptopentane-3: 4: 5-triol	124	2.09	+0.342±0.1367	+0.215±0.0471	—	—	0.289±0.0629	—	43	47	0.90
	1: 2-dimercaptohexane-3: 4: 5: 6-tetrol	162	2.21	+0.007±0.2083	−0.088±0.1337	−0.507±0.1279	−0.538±0.1087	−0.022±0.0826	−1.36	0	59	0
	δ-mercapto-γ-valerothiolactone	160	2.20	+0.208±0.0500	+0.067±0.0904	−0.112±0.0651	−0.421±0.1611	0.196±0.0615	−1.38	28	111	0.25
G	Nil	—	—	−0.010±0.0479	−0.009±0.0107	−0.336±0.0750	—	—	—	—	—	—
	Dimercaprol	160	2.20	+0.611±0.0686	+0.683±0.0377	+0.366±0.0260	—	0.672±0.0418	—	100	100	1.00
	Triacetyl dimercaprol	160	2.20	+0.623±0.2396	+0.221±0.1252	—	—	0.432±0.0793	—	62	62	1.00
	1: 2-dimercaptopropionic acid	160	2.20	+0.174±0.0498	+0.185±0.0593	+0.027±0.0238	—	0.247±0.0349	—	43	167	0.26
	1: 6-hexanedithiol	160	2.20	−0.190±0.0473	−0.317±0.0870	−0.459±0.0482	—	−0.204±0.0410	—	Potentiation	—	—

Discussion

Survival time has not been extensively used in order to measure the effects of drugs in biological work. Bülbring (1937) showed that extracts of adrenal cortex could be assayed by the increase in survival time produced in adrenalectomized drakes. Vogt (1943) showed that this was also true for adrenalectomized rats kept at a low environmental temperature. Both showed that the untransformed survival times of treated animals were approximately linearly related to the logarithm of the dose of cortical extract, but the data of Bülbring do not exclude the possibility that the log survival time is as, or more nearly, linearly related to the log dose of extract. Box and Cullumbine (1947), using mustard gas and phosgene and measuring the dose as the product of the concentration of the gas and the time of exposure, found that the reciprocals of the survival times were normally distributed and linearly related to the dose of gas and that the logarithms of the survival times were not. They discussed the merits of using survival time in assays of antidotes to poisons which produce death but no other easily measured response. Withell (1942) showed that the log survival times of micro-organisms poisoned with a given concentration of bactericides were normally distributed, but apparently did not attempt to relate the mean survival time of the organisms to the concentration of bactericide.

With the two arsenicals and two species used in this work the logarithm of the survival time was within a limited range approximately linearly related to the logarithm of the dose of arsenical; the logarithms of the individual survival times for any one dose of poison were more nearly normally distributed than were the untransformed survival times or the reciprocals of the survival times, and the standard deviations of the individual values were roughly constant and independent of the dose of poison. In animals poisoned with phenylarsenoxide the increment in log survival time produced by dimercaprol was linearly related to the logarithm of the dose of dimercaprol. In view of the widely differing conditions of these various uses of survival time, it is perhaps not surprising that the distributions differ greatly and are not all amenable to the same transformations. Data obtained under other conditions are necessary before generalizations can usefully be attempted.

From the data presented, an attempt can be made to deduce how much poison is inactivated by dimercaprol in the body. Table VII shows what doses of phenylarsenoxide and dimercaprol are required to give certain fixed survival times, according to the regression equations derived from the data. If the survival time of the rats is regarded as depending entirely on the amount of phenylarsenoxide used in the absence of dimercaprol, then the excess necessary to give the same survival time in the presence of dimercaprol can be regarded as the amount neutralized by the dimercaprol. Such amounts are shown in column 4 of Table VII, and are expressed in column 5 as a percentage of the amount of dimercaprol given. It appears that the efficiency of the dimercaprol, as judged by the amount of phenylarsenoxide neutralized per molecule, increases with the dose of dimercaprol to a maximum at about 160 μM per kg. and then remains steady at a level depending on the amount of phenylarsenoxide available. Not much significance need be attached to the values over 100 per cent in the early part of the Table. These values are calculated from two regression equations, in both near or beyond the extremities of the observed values, where the sampling errors of the estimates are largest, the assumption of linearity is most doubtful, and the effect of transforming back from a log dose magnifies discrepancies. In practice, no instance has been observed where the increment in survival time produced by dimercaprol was larger than could be

TABLE VII

CALCULATED DOSES OF PHENYLARSENOXIDE AND DIMERCAPROL NECESSARY TO PRODUCE CONSTANT SURVIVAL TIMES

Survival time. Hours	Dose of phenylarsenoxide, μM/kg.	Dose of dimercaprol. μM/kg.	Phenylarsenoxide neutralized by dimercaprol, μM/kg.	Phenylarsenoxide neutralized, mols per 100 mols dimercaprol
1	108	0	—	—
1	123	40	15	37.5
1	209	80	101	126
1	(359)	(160)	(251)	(157)
1	(481)	(240)	(373)	(155)
4	45	0	—	—
4	51	40	6	15
4	87	80	42	52.5
4	149	160	104	65
4	200	240	155	64.5
4	245	320	200	62.5
4	288	400	243	61
10	(25)	(0)	—	—
10	(28)	(40)	(3)	(7.5)
10	48	80	23	29
10	83	160	63	39.5
10	111	240	86	36

Figures in parentheses are outside the range of experimental observations. The calculation of these figures is described in the text.

accounted for by the inactivation of the equimolar quantity of phenylarsenoxide. Nevertheless, in these conditions (near optimal amounts of dimercaprol and excess of phenylarsenoxide) the efficiency of the antidote does approach very close to 100 per cent of what is chemically possible. Figures similarly calculated for oxophenarsine at optimal dimercaprol levels are about 90 per cent at 1 hour, 40 per cent at 4 hours, and 20 per cent at 10 hours; so that although the total amounts of arsenic in the body are larger, dimercaprol appears to be somewhat less effective against this poison.

Hogan and Eagle (1944) and Chance, Crawford, and Levvy (1945) showed that in rabbits poisoned with phenylarsenoxide or oxophenarsine, phenylarsenoxide was excreted much more slowly than oxophenarsine. Chance and Levvy (1947) showed that dimercaprol given to phenylarsenoxide poisoned rabbits increased the excretion of arsenic tenfold, and in oxophenarsine poisoned rabbits increased the excretion of arsenic only 2.5 times. Peters and Stocken (1947) showed that the compound formed *in vitro* between oxophenarsine and dimercaprol (4-hydroxymethyl-2-(3′-amino-4′-hydroxyphenyl)-1 : 3-dithia-2-arsa*cyclo*pentane) was much more toxic than oxophenarsine, and that treatment with dimercaprol prevented toxic effects of the oxophenarsine-dimercaprol compound. In unpublished experiments (Weatherall, 1949) it was shown that when phenylarsenoxide and dimercaprol were mixed in equivalent amounts in aqueous solution a white precipitate formed: and when a suspension of this precipitate containing 3 × LD50 of phenylarsenoxide was injected intramuscularly animals showed no ill effects.

The toxicity of the oxophenarsine-dimercaprol compound and the small increase in the excretion of arsenic after dimercaprol in oxophenarsine poisoned animals, compared with the apparent harmlessness of the phenylarsenoxide-dimercaprol mixture and the much increased excretion of arsenic after dimercaprol in phenylarsenoxide poisoned animals probably explains why dimercaprol is less efficient in preventing oxophenarsine poisoning than in phenylarsenoxide poisoning.

The other dithiols investigated fall into five chemical classes: 1 : 2-dithiol derivatives of polyhydric alcohols, oxygen ethers of dimercaprol, 1 : 2-dithiols containing a carboxyl group, αω-dithiols and acetylated dithiols. The first group are compounds with the general formula $CH_2SH.CHSH.(CHOH)_nCH_2OH$. In this series (exp. *F*, Table VI) activity was maximal when $n = 0$, i.e., in dimercaprol, and fell off as n increased, until 1 : 2-dimercaptohexane-3 : 4 : 5 : 6-tetrol ($n = 4$) had no activity. This decrease accompanies a decrease in lipoid solubility. The second group were dimercaprol ethers of mannitol, glucose, or tetramethylglucose (exp. *E*, Table VI). The former two were moderately active, but the latter showed little activity. 1 : 2-Dimercaptopropionic acid in the third group was about one and half times as active as δ-mercapto-γ-valerothiolactone. Possibly the lactone ring in this compound did not break *in vivo*, so that the substance contained only one active –SH group. However, Weatherall (1949) showed that a dose of this compound equivalent to 1.54 times the LD99 of oxophenarsine completely prevented mice from dying even when given 80 min. after the LD99 of oxophenarsine. This suggests that the substance does not act *in vivo* as a monothiol, because other monothiols, cysteine and glutathione, are useless in preventing death in oxophenarsine poisoned animals when given only 5 min. after the oxophenarsine (Eagle, Magnuson, and Fleischman, 1946). The only αω-dithiol used in this work potentiated poisoning by phenylarsenoxide. This was surprising because Whittaker (1947) showed that 1 : 6-hexanedithiol reactivated pigeon brain pyruvate oxidase poisoned with lewisite when added ten minutes after the lewisite; Thomson, Savit, and Goldwasser (1947) showed that skin exposed to lewisite was decontaminated by 1 : 6-hexanedithiol, although less efficiently than by dimercaprol, and Kensler, Abels, and Rhoads (1946) showed that it was effective in the treatment of arsine poisoning, again less so than dimercaprol. On the other hand, Weatherall (1949) found that other αω-dithiols (1 : 4-dithiothreitol and 1 : 4-dithioerythritol) accelerated death in oxophenarsine poisoned mice and the former caused a large increase in the mortality from sublethal doses of oxophenarsine. It would be interesting to know more about the changes in survival time produced by other doses of 1 : 6-hexanedithiol in phenylarsenoxide poisoned rats. Possibly larger doses would have increased the survival time. Triacetyldimercaprol, the only acetylated dithiol tested, was about as active as dimercaprol glucoside. This is consistent with the results of mortality experiments (Weatherall, 1949).

For practical purposes the ratio of therapeutic activity to toxicity is more important than the absolute therapeutic activity per gramme or per gramme-molecule. As an increase in LD50 represents a reduction in toxicity, the efficiency of these substances has been expressed as the ratio of the anti-arsenical activity and the toxicity, both measured with unit activity as that of dimercaprol, as shown in the last column of Table VI. Only the sugar ethers of dimercaprol surpass dimercaprol when considered in this way, and of the sugar ethers the glucoside is substantially the best.

No attempt has so far been made to observe the effect of delaying treatment with dithiols on the relation between survival time and the dose of dithiol, nor to extend the method used in these experiments to determine the relation between other poisons and survival time, or the effects of dithiols on poisoning by other substances.

A substance which increases the survival time of experimental animals is not necessarily an effective therapeutic agent in either animals or men. Weatherall (1949) has shown that all the substances discussed here with the exception of 1:2-dimercaptohexane-3:4:5:6-tetrol, 1:2-dimercaptopropionic acid, and 1:6-hexanedithiol, which he did not test, reduced the mortality in oxophenarsine poisoned mice, and the compounds which he found most efficient in preventing death in oxophenarsine poisoned mice are those which in the experiments now reported have been found most efficient in prolonging the survival time of phenylarsenoxide poisoned rats. In the present work the efficiency of the dithiol has been measured only when it has been given immediately after the poison. It is likely that the relative efficiency of different dithiols will differ when they are used in the delayed treatment of acute poisoning or in chronic poisoning. It is, however, unlikely that a dithiol which has no activity when given immediately after the poison, at a time when the poison is circulating freely, will have appreciable effect in chronic poisoning when the poison has been fixed by the tissues. The present procedure, therefore, provides a test for excluding ineffective substances, and a quantitative method for differentiating between substances with almost equal activity and for studying changes in activity produced by changes in chemical structure.

Summary

1. Lethal doses of phenylarsenoxide and oxophenarsine have been injected intramuscularly into rats and mice and the survival times have been measured.

2. Over the range of doses used the log survival time was as a rule linearly related to the log dose and at least with phenylarsenoxide to the temperature of the room also.

3. Some poisoned animals were immediately injected intramuscularly with dimercaprol. Those treated with dimercaprol lived longer than those untreated.

4. For a given dose of dimercaprol, the relation between the log dose of phenylarsenoxide and the log survival time was linear and the slope was the same as in the absence of dimercaprol.

5. The mean increment in log survival time produced by different doses of dimercaprol in phenylarsenoxide poisoned rats was linearly related to the log dose of dimercaprol. This relation was not grossly affected by small differences in environmental temperature.

6. From a consideration of the two regressions, it appeared that the amount of phenylarsenoxide inactivated by dimercaprol increased to a maximum when the dose of dimercaprol was about 160 μM/kg. and was greater with large doses of phenylarsenoxide.

7. Similar data are presented for oxophenarsine and dimercaprol in rats and mice. The results are less comprehensive, but suggest that in very small doses dimercaprol can accelerate death in oxophenarsine poisoned mice, and that with larger doses of dimercaprol the relationship resembles that to phenylarsenoxide.

8. In the presence of dithiols, the relation between the log dose of phenylarsenoxide and the log survival time was linear but not always parallel to the line for phenylarsenoxide alone. The differences were not large and approximate comparisons of the activity of ten dithiols and related substances have been made and are discussed. None of the substances was more active than dimercaprol.

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DIMERCAPROL AND THE BILIARY EXCRETION OF LEAD IN RABBITS

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Ginsburg and Weatherall (1948) showed that dimercaprol injected intramuscularly in rabbits shortly after lead acetate had been injected intravenously increased the amount of lead in the alimentary canal and particularly in its contents, and decreased the amount in the liver. The concentration of lead in bile obtained from the gall bladder was very variable and did not provide good evidence about the part played by biliary excretion in this redistribution. The present experiments were directed to settling this point.

Methods

The procedures used in preparing solutions, administering and estimating lead by means of the tracer Ph^{212} (thorium B) have already been described (Ginsburg and Weatherall, 1948).

In order to collect samples of bile, rabbits weighing 2.5–2.9 kg. were anaesthetized with intravenous pentobarbitone or ether and cannulae were tied into the trachea, the internal carotid artery and the common bile duct. The cystic duct was tied. The arterial blood pressure was recorded, and artificial respiration was applied if necessary. Lead acetate (2.07 mg. Pb/kg.) containing about 200 microcuries of thorium B was then given intravenously and bile was collected for the next four periods of one hour, and their content of lead estimated. In some rabbits, dimercaprol (12.5 mg./kg.) in 66 per cent (v/v) aqueous propylene glycol (0.25 mg./kg.) was injected intramuscularly two hours after the lead acetate, and into the rest 66 per cent propylene glycol alone. This dose is one-quarter of that previously used for the initial injection, and has been reduced because it is doubtful whether rabbits already subjected to anaesthesia and operative trauma would tolerate much larger amounts. In one rabbit (No. 355) the dose of dimercaprol was 4 mg./kg. After 4 hours the rabbits were killed with a large dose of pentobarbitone and certain tissues were taken for the estimation of the lead in them.

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Results and Discussion

The results are shown in Table 1 and Fig. 1. When dimercaprol was not administered, the highest

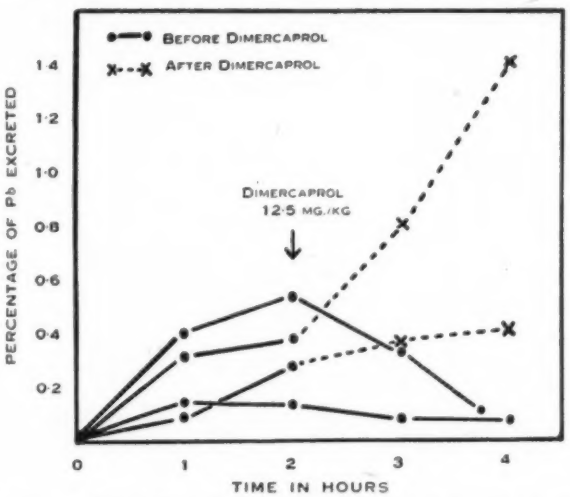

FIG. 1.—The effect of dimercaprol on the excretion of lead in bile. Abscissa: time in hours after the intravenous administration of lead acetate (2.07 mg. Pb/kg.). Ordinate: percentage of administered lead excreted per hour in bile.

concentration of lead in the bile occurred in the second hour and later samples contained less. When dimercaprol was injected after the second hour, even in a dose as small as 4 mg./kg., the concentration continued to rise, except in the rabbit anaesthetized with ether. This rabbit was in poor condition and died before the end of the third hour and it is unlikely that the lack of response was due directly to the different anaesthetic. In all these experiments there was a progressive decline in the blood pressure without striking changes referable to the lead acetate or dimercaprol. There was no indication that the increased excretion after dimercaprol was due to circulatory improvement.

TABLE I

THE EFFECT OF DIMERCAPROL ON THE FATE OF LEAD IN ANAESTHETIZED RABBITS WITH CANNULATED BILE DUCTS, AFTER THE INTRAVENOUS ADMINISTRATION OF LEAD ACETATE (2.07 mg. Pb/kg.)

Rabbit number:— / Weight and sex:—	323† 2.9 kg.♂			353 2.5 kg♂			355 2.9 kg.♀			317 2.6 kg.♂			345 2.5 kg.♂			356* 2.5 kg.♀		
Anaesthetic:—	Pentobarbitone															Ether		
Dose of dimercaprol mg./kg.	Nil			Nil			4.0			12.5			12.5			12.5		
	Bile vol. (ml.)	% of dose	μg. Pb per ml.	Bile vol. (ml.)	% of dose	μg. Pb per ml.	Bile vol. (ml.)	% of dose	μg. Pb per ml.	Bile vol. (ml.)	% of dose	μg. Pb per ml.	Bile vol. (ml.)	% of dose	μg. Pb per ml.	Bile vol. (ml.)	% of dose	μg. Pb per ml.
Hepatic bile 1st hr. ...	12.0	0.40	2.0	8.7	0.15	0.78	9.3	0.11	0.72	8.1	0.12	0.88	3.6	0.34	4.8	3.6	0.04	0.53
,, ,, 2nd ,, ...	9.0	0.54	3.7	6.5	0.15	1.0	9.2	0.22	1.4	7.2	0.24	1.9	3.8	0.38	5.2	6.0	0.16	1.4
,, ,, 3rd ,, ...	6.9	0.34	3.0	6.3	0.08	0.57	8.2	0.23	1.6	6.3	0.36	3.2	3.7	0.83	11.6	3.3	0.04	0.85
,, ,, 4th ,, ...	2.5	0.08	1.9	6.3	0.08	0.61	8.2	0.38	2.8	3.8	0.41	6.1	3.4	1.4	21	—	—	—
,, ,, Total ...	—	1.36	—	—	0.46	—	—	0.94	—	—	1.13	—	—	2.95	—	—	0.24	—
	% dose		μg. Pb per gm.	% dose		μg. Pb per gm.	% dose		μg. Pb per gm.	% dose		μg. Pb per gm.	% dose		μg. Pb per gm.	% dose		μg. Pb per gm.
Liver at death	43		31	33		31	53		29	71		67	36		40	32		25
Small intestine at death ...	2.4		2.0	0.21		0.17	2.0		1.4	0.90		0.88	1.1		1.3	0.74		0.70
Small intestine contents at death	0.38		—	0.12		0.70	0.49		3.5	0.66		1.5	0.33		1.7	0.09		0.02
Blood	3.1		—	5.0		1.5	—		0.90	—		—	5.1		1.5	—		—

† Died 3 hrs. 45 min. after injection of lead.

* Died 2 hrs. 40 min. after injection of lead.

The average amount of lead in the liver was not reduced by dimercaprol in the rabbits and the amount of lead in the small intestine in one of the control animals was much higher than any previously observed by us. As the bile duct was ligated, this suggests that lead is also excreted directly by at least some part of the alimentary canal. The high value was associated with considerably more handling of the gut than usual in the operative procedure and was perhaps due to consequent hyperaemia. The present results for tissues are very variable and cannot be compared with the findings in normal rabbits.

Summary

Dimercaprol increases the excretion of lead in the bile of anaesthetized rabbits.

We are indebted to Miss Irene Munro for technical assistance, and to the Therapeutic Research Corporation for a personal grant to one of us (M.G.), and to the Medical Research Council for a grant for expenses to the other of us (M.W.).

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pA_x AND COMPETITIVE DRUG ANTAGONISM

BY

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In a recent paper Guarino and Bovet (1949) have studied the use of the measure pA for expressing intensity of drug antagonism. pA_x has been defined (Schild, 1947) as the negative logarithm of the molar concentration of an antagonist which reduces the effect of a multiple dose, x, of a stimulant drug to that of a single dose. Guarino and Bovet have pointed out that this definition does not specify concentration of active drug, and therefore by implication assumes that pA is independent of the initial concentration of active drug used. In studying the antagonism between acetylcholine and flaxedil* on the frog's rectus abdominis, however, they found that pA was not entirely independent of the initial concentration of acetylcholine used. For instance, an increase in the concentration of acetylcholine of 1.5 log units produced a decrease of pA_2 of 0.53 units. Guarino and Bovet then proceeded to evolve a new formula for the quantitative relations of antagonistic drugs whereby pA would be dependent on the concentration of active drug in competitive antagonism.

This interesting paper raises several issues connected with the use of pA and the definition of competitive drug antagonism. I propose to discuss the following points:

1. Conditions under which pA is independent of concentration of active drug.
2. Quantitative relations of antagonistic drugs.
3. The practical use of pA.

Dependence of pA on Height of Contraction. Types of Concentration-Action Curves

It is well known that concentration-action curves plotted on a logarithmic scale, first in the absence of an antagonist and then in the presence of antagonist, are frequently parallel. This experimental fact forms the basis for Gaddum's formula (1937) for drug antagonism and it is also at the basis of the pA measure, since parallelism on a log abscissa implies that pA is independent of height of contraction (and hence of concentration of active drug). A simple way of finding whether pA is applicable is thus to draw concentration-action curves on a log scale with and without antagonist. This method may also be used to determine pA values.

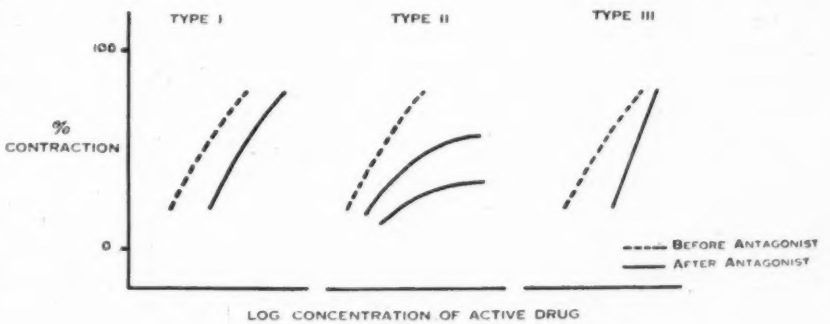

FIG. 1.—Three types of concentration-action curves.

Concentration-action curves have also been described, however, which are not parallel when plotted on a logarithmic scale, and where pA in consequence is dependent on the height of contraction. Amongst these, two types emerge. One type (type II of Fig. 1) has been described by Zadina (1947) with the following two characteristic properties: the concentration-action curves become progressively flatter and their maxima decline as the concentration of antagonistic drug is increased. Examples are: histamine-harmine on guinea-pig ileum (Zadina, 1947), acetylcholine-magnesium on guinea-pig ileum (Zadina and Kriz, 1948), acetylcholine octyltrimethylammonium on rat's intestine (Clark and Raventos, 1937). Particularly in Zadina's careful experiments it is perfectly clear that not only the slopes but also the maxima of the curves are decreased. These results are presumably due to some sort of non-competitive antagonism, since the effects of the antagonist cannot be completely

* 1:2:3—Tri-β-diethylaminoethoxybenzene triethiodide.

reversed. Such curves might occur, for example, if a constant number of contractile elements (or " receptors ") were put out of action by a given concentration of antagonist. This would have the effect of decreasing both slopes and maxima.

Lastly there are curves of type III (Fig. 1), for example Guarino and Bovet's curves obtained with acetylcholine-flaxedil on the frog's rectus; here the concentration-action curve with antagonist is steeper than without. It was in order to account for these results that Guarino and Bovet suggested a new formula for competitive drug antagonism.

Quantitative Relations of Competitive Antagonists

Gaddum (1937) and Guarino and Bovet (1949) have suggested formulae to express quantitatively the relations of antagonistic drugs competing for receptors. The two formulae are incompatible; it can be shown, in fact, that according to one of them pA is independent of concentration of active drug, whilst according to the other it increases as the negative logarithm of concentration of active drug. Since the matter is of general interest I shall try, in the following, to point out the differences underlying these two formulations of competitive antagonism.

Gaddum's formula is based on the assumption that drug and antagonist compete for free receptors on the cell surface according to a simple mass action law, and that at equilibrium the number of drug-receptor combinations is equal to the number of drug-receptor dissociations and similarly for the antagonist. Contraction of the muscle is supposed to be proportional to the average number of receptors occupied by the active drug. With these assumptions a formula can be derived which relates percentage contraction y to concentration A of stimulant drug and B of antagonistic drug:

$$\frac{y}{100-y} = K_1 A = \frac{K_1 x A}{K_2 B_x + 1} \quad \ldots\ldots\ldots\ldots\ldots (1)$$

$$\therefore K_2 B_x = x - 1.$$

A similar equation is obtained by applying Langmuir's adsorption isotherm to the case of two gases competing for the same surface (Taylor, 1931).

Guarino and Bovet's formula is derived as follows:

Assuming concentration A of a drug to produce effect y, and concentration $2A$ to produce a bigger effect, what concentration of antagonist is required to reduce the effect of $2A$ to that of A? To simplify the argument it is assumed that the antagonist (" false drug ") has the same affinity for receptors as the active (" true ") drug has. The answer is that the concentration of false drug required is equal to that of true drug, namely $2B = 2A$, since in this way the chances of true drug reacting with receptors will be halved and hence its action will be reduced to that of A. Generalizing this argument, if the concentration of true drug is increased to xA, then the amount of false drug required to reduce this effect to that of A must be such that the total amount of true and false drug present in the solution is x times the amount of true drug present. This leads to the following general formula:

$$K_2 B_x = x(x-1) K_1 A \quad \ldots\ldots\ldots\ldots\ldots (2)$$

where B_x is concentration of antagonist required to reduce the effect of concentration xA of stimulant drug to that of A.

In non-competitive antagonism this formula is assumed to reduce to simple proportionality, namely:

$$K_2 B_x = x K_1 A \quad \ldots\ldots\ldots\ldots\ldots (3)$$

This formula, incidentally, was originally used by Clark (1926) to formulate the antagonism acetylcholine-atropine, K_1/K_2, depending on the effect produced.

That the three equations are incompatible may be shown by transforming each to give the antagonist ratio B_{x_2}/B_{x_1}, required to balance an increase of active drug from x_1-fold to x_2-fold.

From equation (1)

$$\frac{B_{x_2}}{B_{x_1}} = \frac{x_2 - 1}{x_1 - 1}$$

From equation (2)

$$\frac{B_{x_2}}{B_{x_1}} = \frac{x_2(x_2-1)}{x_1(x_1-1)}$$

From equation (3)

$$\frac{B_{x_2}}{B_{x_1}} = \frac{x_2}{x_1}$$

In this way the applicability of one or the other equation may be tested. For example, in order to balance an increase of active drug from 2-fold to 10-fold the antagonistic drug would have to be increased 9 times, 45 times, or 5 times respectively, according to the three equations. Unfortunately, this test is not very fruitful, since all sorts of ratios seem to occur; for example, a B_{10}/B_2 ratio of 5 was found for acetylcholine-atropine, 10 for histamine-benadryl (Schild, 1947), and 21 for acetylcholine-flaxedil (Guarino and Bovet, 1949).

With regard to pA the following difference exists between equations (1) and (2). From (1), $B_x = x - 1/K_2$. Therefore B_x is not a function of A. Since $pA_x = -\log B_x$, it also is not a function of A. Hence whenever equation (1) applies pA_x must be independent of concentration of stimulant drug.

From (2) B_x is proportional to A. Therefore pA is inversely related to concentration of stimulant

drug. Thus an increase in the concentration of stimulant drug by one log unit should cause pA to diminish by one unit.

In Guarino and Bovet's experiments pA did not decrease to the extent required by their theory, but they regard equation (2) as a limiting case of perfect competitive antagonism which has not yet been shown to occur.

Guarino and Bovet's assumptions differ in two respects from the assumptions underlying, e.g., equation (1).

(i) It is assumed that if to a given concentration of true drug a quantity of false drug be added to increase total concentration x-fold, then the chances of true drug reacting with receptors would be reduced to $1/x$. By equation (1) this would only apply if all receptors were initially occupied. Otherwise the addition of antagonist would increase the total number of receptors occupied and the chances of reacting would be reduced to $1/x$ of the new total and not of the original.

(ii) It is assumed that reducing the chances of receptor combination to $1/x$ by false drug has the same effect as decreasing the concentration of true drug to $1/x$. By equation (1) this would apply approximately when a few receptors were occupied, but would not apply when most receptors were occupied. This may be illustrated as follows: Assume a sufficient concentration of true drug present to produce an almost maximal effect. If this concentration were halved the effect would be only slightly decreased since most receptors would still be occupied. If, however, an equivalent amount of false drug were added each receptor would have an equal chance of being occupied by one or the other and the effect would be halved.

In conclusion Guarino and Bovet's assumptions do not appear to be based on mass action competition for receptors in the sense that drug or antagonist occupy a number of receptors for a finite time to the exclusion of each other. Langmuir's treatment is based on the assumption that after condensation of a molecule of the gas owing to adsorption on an "elementary space" a finite interval elapses before it escapes again from the surface. This notion gives a clear physical meaning to the term competition, which is absent in Guarino and Bovet's hypothesis.

The Practical Use of pA

Methods of measuring activity of antagonistic drugs may be divided into comparative and non-comparative methods and into methods in which the antagonist is injected first (preventive) and methods in which the active drug is injected first (curative). pA belongs to the group of non-comparative and preventive methods and in this way its field of usefulness is limited. For example, it is difficult to use a preventive method quantitatively in the whole animal since this requires producing a constant *concentration* of antagonist in the blood stream; on the other hand it is often possible to produce a constant *effect* with the active drug and reverse it by means of an antagonist (e.g., Ing, Dawes, and Wajda, 1945). The main disadvantage of pA is its variability, which is greater than that of an assay relying on a comparison with another antagonistic drug. For this reason comparative assays are likely to be preferred for routine work, but for the purposes of a general scale an absolute method and one in which the effect of time and concentration can be accurately assessed is preferable (Schild, 1947).

One way of decreasing variability is to define in great detail the conditions of the experiment. There is little purpose, however, in introducing arbitrary limitations which do not add to our information, unless some important systematic error is thereby avoided. For example, it is probably not necessary to choose a uniform strain of guinea-pigs since pA does not appear to vary systematically from one laboratory to another (cf. results of Reuse (1948) and Schild (1947)).

In an antagonist assay on an isolated preparation the following variables may determine the effect: experimental preparation, concentration of antagonistic drug, time of contact with antagonistic drug, concentration of active drug, effect produced by active drug alone, composition of Ringer's fluid, temperature, etc. In measurements of pA only the first two or three are defined. By implication the others may be assumed to introduce no important systematic variation.

In practice a compromise must be found between having too many arbitrary limitations and excessive variability. For example, in the method of Miller, Becker, and Tainter (1948) the concentration of active drug is fixed. This introduces an arbitrary limitation, but it certainly helps to reduce variability. In the case of pA neither concentration of active drug nor effect produced by active drug is defined, hence the assumption is that for practical purposes pA is independent of these variables. This is obviously not true when dealing with concentration-action relations of type II and III above, and I would suggest that in such cases pA be measured when the effect is 50 per cent of the maximal. In this way pA may be used as a practical measure of antagonistic activity even when it is not independent of concentration of active drug.

Summary

1. Three types of concentration-action curves have been described when the effect of a drug on an isolated tissue is plotted against the logarithm of the dose, in the absence and presence of antagonist. Curves of type I remain parallel, those of type II become flatter, and those of type III steeper in the presence of antagonist. Type II is often associated with a lowering of the maximum contraction (Zadina). By definition pA is constant only when curves of type I occur.

2. Guarino and Bovet have recently shown that curves of type III may occur and have suggested a formula which would account for these curves. Gaddum had previously suggested a different formula for competitive drug antagonism, which accounts for curves of type I. The two formulae are contrasted and the conclusion is reached that Guarino and Bovet's formula implies a type of antagonism which does not involve the notion that drug and antagonist compete for and occupy the same receptors according to a law of mass action. The available experimental data are insufficient to distinguish between the two views.

3. Variability may be diminished by defining the height of contraction at which pA should be determined. With curves of type I it is unnecessary thus to define the effect, but in other cases a 50 per cent contraction may be chosen for determining pA.

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SOME PHARMACOLOGICAL PROPERTIES COMMON TO ANTIHISTAMINE COMPOUNDS

BY

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In recent communications (Dutta, 1948; Burn and Dutta, 1948; Dutta, 1949) it has been shown that β-dimethylaminoethyl benzhydryl ether hydrochloride ("Benadryl," Parke, Davis and Co., Ltd.) possesses some pharmacological properties in common with atropine, pethidine, procaine, and quinidine; it produces a fall of body temperature in mice, abolishes the constrictor action of adrenaline and histamine on the perfused vessels of the rabbit's ear, and relieves histamine-induced bronchoconstriction in the guinea-pig. It was also demonstrated that 2-(N-phenyl-N-benzylaminomethyl)iminazoline hydrochloride ("Antistin," Ciba, Ltd., or "Histostab," Boots' Pure Drug Co., Ltd.) shares some of these properties, for it, too, antagonizes the action of adrenaline on the rabbit ear vessels and is only slightly less effective than benadryl in alleviating bronchial constriction in guinea-pigs after the intravenous injection of histamine. The investigation of these substances has since been continued, and in this paper the actions of β-dimethylaminoethyl benzhydryl ether hydrochloride (which will be called benadryl) and 2-(N-phenyl-N-benzylaminomethyl)iminazoline hydrochloride (which will be called antistin) on cardiac and skeletal muscle and on ganglionic transmission are described. The results of an investigation of benadryl on gastric acidity induced by histamine are also presented.

Local anaesthetic action

The majority of the common antihistamine substances are local anaesthetics. This has been shown to be true for benadryl (Leavitt and Code, 1947) and antistin (Brack, 1946) in man and in frogs (Reuse, 1948). Graham (1947) referred to the local anaesthetic action of these substances on guinea-pigs but did not give any details of his findings. In the present investigation the strength of these substances was determined in relation to procaine by the intracutaneous weal method in guinea-pigs described by Bülbring and Wajda (1945). In guinea-pigs the sensitiveness of the different areas of the skin on the back varies considerably. In order to reduce this to a minimum, Somers and Edge (1947) suggested the use of a statistical design of Latin squares for the arrangements between the doses and the sites at which the substances are to be injected. This was adopted. Three animals were used for each comparison. The back of each guinea-pig was divided into 9 squared areas. Procaine, antistin, and benadryl were injected intradermally in three different concentrations (for each substance) on three consecutive days. All these compounds were given at the same dose level on any one day, and each animal received each substance but at different sites. This is shown in Table I. On the first day of the test guinea-pig 1 received low doses of procaine, antistin, and benadryl in the front, middle, and rear part of the back respectively on the left-hand side. The reaction to 6 pin-pricks was

TABLE I

EXPERIMENTAL DESIGN, SHOWING THE POSITION OF THE INJECTIONS ON THE BACK OF THE GUINEA-PIGS

	Areas	Guinea-pigs 1	2	3
1st day—low dose ...	α	P	B	A
	β	A	P	B
	γ	B	A	P
2nd day—middle dose	α	A	P	B
	β	B	A	P
	γ	P	B	A
3rd day—high dose ...	α	B	A	P
	β	P	B	A
	γ	A	P	B

α = front. β = middle. γ = back. P = procaine. A = antistin. B = benadryl.

TABLE II

NUMBER OF PRICKS (OUT OF 36) FAILING TO ELICIT A RESPONSE AFTER INTRACUTANEOUS INJECTION OF PROCAINE, ANTISTIN, AND BENADRYL IN THE GUINEA-PIG

Compounds:	Procaine hydrochloride			Antistin			Benadryl		
Concentration in per cent (w/v):	0.1	0.25	1.0	0.04	0.1	0.25	0.04	0.1	0.25
Pig 1	8	7	30	6	7	29	5	8	31
,, 2	13	20	26	4	20	29	7	26	24
,, 3	4	21	36	8	16	31	12	20	36
,, 4	5	18	22	3	9	5	3	18	15
,, 5	5	16	31	4	15	35	4	20	24
,, 6	10	7	36	11	17	12	7	21	36
Mean:	7.5	15	30	6	14	23.5	6.3	19.0	27.6

determined at each site every five minutes. The total number of times a prick failed to produce a response in the course of half an hour, out of a possible 36, indicated the degree of anaesthesia in that particular spot. Next, guinea-pig 2 was injected with benadryl in the front, procaine in the middle. and antistin in the rear part on the left squared areas as before and so on. On the second day the injections were given in the middle areas and on the third day on the right-hand side squares. Two complete series of experiments were carried out with 6 guinea-pigs.

There were 54 individual comparisons at different sites on 6 guinea-pigs. The results are summarized in Table II. Each of the figures in Table II (except at the bottom line) represents the total number of pricks, out of 36, which failed to elicit a response at a given site during the half-hour observation. The mean values for the six experiments are given at the base line. Variation in the sensitiveness of the different areas of the skin is evident if some of the individual figures are examined. For instance, antistin in 0.25 per cent concentration produced less effect than in 0.1 per cent in guinea-pig 4. Similar results were also obtained from guinea-pig 6. The higher concentration of benadryl caused a lower degree of anaesthesia than the lower concentration of the drug on guinea-pig 4. These are the extreme examples.

When the mean figures indicating the degree of anaesthesia for any one of the given substances (shown in Table II) are plotted as ordinates against its logarithmic concentrations as abscissae, a linear relationship is obtained. This is illustrated in Fig. 1. The slopes of the lines for procaine, antistin, and benadryl are approximately parallel within the doses mentioned above. This parallelism did not exist when higher concentrations of antistin or lower doses of benadryl were used. At dose levels where all the three substances prevented 50 per cent of the skin reactions, benadryl was 3.2 times and antistin 2.3 times as potent as procaine.

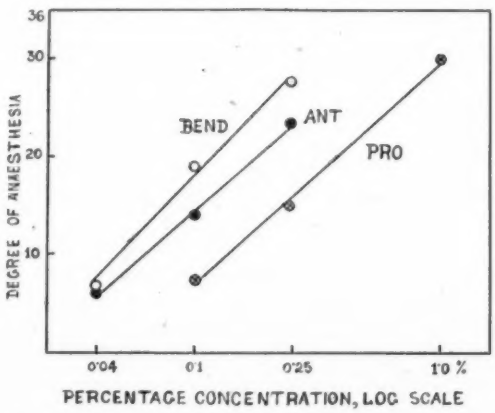
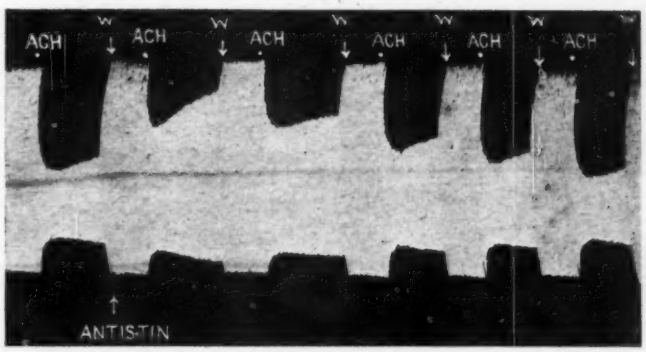


FIG. 1.—Intracutaneous weal in guinea-pigs. The graph shows the relation between the concentration of the local anaesthetic (abscissae) and the number of times there was no response to a prick; total 36 pricks in 30 min. (ordinates). Each point represents the mean of six observations. BEND = benadryl; ANT = antistin; PRO = procaine hydrochloride.

Antagonism to acetylcholine on cardiac muscle

It is known that both antistin and benadryl antagonize certain actions of acetylcholine, and consequently whether they would also antagonize acetylcholine on the isolated rabbit's auricles beating in oxygenated Ringer-Locke's solution at 29° C. was investigated. Both these substances reduced



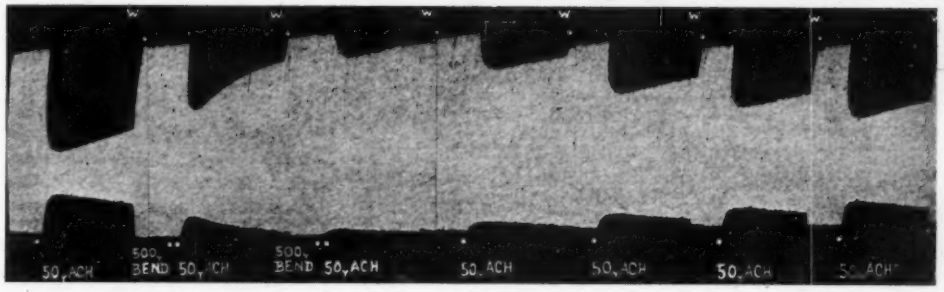


FIG. 2.—Isolated rabbit auricles. Ringer-Locke's solution at 29° C.; 50 ml. bath. *Upper record:* Depression caused by 10 μg. acetylcholine for 2 min. (at the end of which the solution was changed, W) at 6-min. intervals. The second dose of acetylcholine was preceded 1 min. before by 500 μg. antistin. The inhibition due to acetylcholine was reduced. *Lower record:* Depression caused by 50 μg. acetylcholine for 2 min. (at the end of which the solution was changed, W) at 5-min. intervals; 500 μg. benadryl was given 40 sec. before both the second and third doses of acetylcholine. The effect of acetylcholine was greatly diminished in the presence of benadryl.

the depression caused by acetylcholine on the auricular contractions. Repeated washings were required before the inhibition produced by acetylcholine was fully recovered. The experiments are illustrated in Fig. 2.

Effect on the refractory period of the cardiac muscle

Dawes (1946) stated that the quinidine-like action of a substance is associated with its ability to oppose the action of acetylcholine. Benadryl and antistin were, therefore, tested on the isolated rabbit's auricles stimulated electrically in order to determine whether their power to antagonize the action of acetylcholine was associated with a quinidine-like action. The method followed was that of Dawes. The isolated rabbit's auricles were suspended in a 100 ml. bath of oxygenated Ringer-Locke's solution (containing double the usual amount of glucose) at 29° C. The effect of benadryl is illustrated in Fig. 3. Before addition of benadryl to the bath the auricles were able to follow 252 stimuli per min. but not 260 per min. or more. After 100 μg. benadryl had been added to the bath the auricles first followed stimulation at 184 per min. but not at 204 per min. and finally followed 200 per min. Benadryl, therefore, in a concentration 1 in 1,000,000 reduced the maximal rate by 20.6 per cent. The relation between the percentage decrease in the maximal rate at which the auricles could follow electrical stimuli and the logarithm of concentrations is linear. Similar results were obtained with antistin. The results of all the experiments are summarized in Table III. Unlike antistin, the effect of benadryl on the auricles is more persistent; therefore, when its action was compared with that of quinidine, not more than two doses of each of the substances were used on any individual preparation. Benadryl and antistin are about twice as active as quinidine at the level at which the three substances reduce the maximal rate by 15 per cent.

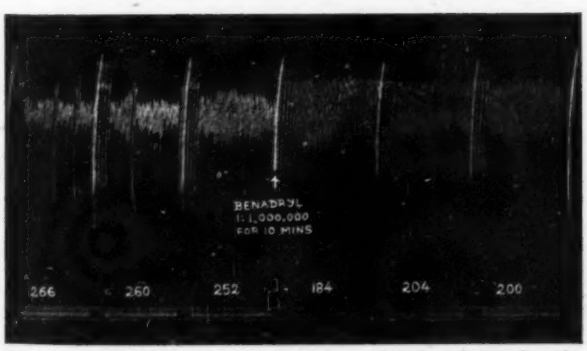


FIG. 3.—Isolated rabbit auricles stimulated by break induction shocks. Ringer-Locke's solution at 29° C., bath 100 ml. The auricles fail to follow up to 260 stimuli per min., but follow 252 per min. After 10 min. exposure to 1: 1,000,000 benadryl at the arrow (the drum was stopped for 9 min.) the auricles follow up to 200 but fail to follow 204 stimuli per min.

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TABLE III

COMPARISON OF BENADRYL, ANTISTIN, AND QUINIDINE ON THE "DRIVEN" RABBIT AURICLE

Exp. No.	Substances	Conc.	Percentage reduction in the maximal rate at which the auricles followed
4	Benadryl	10^{-6}	10.5
	,,	2×10^{-6}	16.6
	,,	4×10^{-6}	23.0
	,,	8×10^{-6}	27.5
5	Benadryl	10^{-6}	20.6
	,,	2×10^{-6}	39,3
	Quinidine HCl	10^{-6}	8.2
	,, ,,	2×10^{-6}	29.0
6	Benadryl	4×10^{-6}	10.0
	,,	8×10^{-6}	14.1
		1.6×10^{-6}	23.0
7	Benadryl	10^{-6}	13.1
	,,	2×10^{-6}	20.2
	Quinidine HCl	10^{-6}	8.4
	,, ,,	2×10^{-6}	14.0
8	Quinidine HCl	10^{-6}	10.5
	,, ,,	2×10^{-6}	18.2
	Benadryl	2×10^{-6}	22.2
	,,	4×10^{-6}	30.0
9	Antistin HCl	10^{-6}	6.9
	,, ,,	2×10^{-6}	13.6
	,, ,,	4×10^{-6}	29.6
	Quinidine HCl	2×10^{-6}	12.5
	,, ,,	4×10^{-6}	17.0
		8×10^{-6}	27.0
10	Antistin HCl	10^{-6}	3.2
	,, ,,	2×10^{-6}	16.0
	,, ,,	4×10^{-6}	21.5
	Quinidine HCl	2×10^{-6}	10.9
	,, ,,	4×10^{-6}	12.5
	,, ,,	8×10^{-6}	15.3
11	Antistin HCl	10^{-6}	3.6
	,, ,,	2×10^{-6}	6.4
	,, ,,	2×10^{-6}	12.8
	Quinidine HCl	2×10^{-6}	3.9
	,, ,,	4×10^{-6}	7.0
	,, ,,	8×10^{-6}	12.5
12	Antistin HCl	2×10^{-6}	9.5
	,, ,,	4×10^{-6}	13.7
	,, ,,	8×10^{-6}	16.4
	Quinidine HCl	2×10^{-6}	9.0
	,, ,,	4×10^{-6}	12.5
	,, ,,	8×10^{-6}	17.4

Antagonism to acetylcholine on skeletal muscle

These observations were first made on the skeletal muscle of the frog. A strip from the frog's (*Rana temporaria*) rectus abdominis was suspended in the oxygenated frog-Ringer solution in a 5 ml. bath at room temperature. The solution was replaced every three minutes by the frog-Ringer solution containing a known concentration of acetylcholine which was allowed to act for 90 sec. Benadryl (Fig. 4) reduced the stimulant action of acetylcholine on the muscle. Similar results were obtained with antistin. The response to 10^{-5} acetylcholine was considerably diminished after exposure of the muscle to 10^{-4} antistin for 3 min.

FIG. 4.—Frog's rectus abdominis in frog-Ringer solution at room temperature. The contractions are due to 2×10^{-6} acetylcholine for 90 sec. every 3 min. The contraction at the arrow was preceded by 30 sec. exposure to 2×10^{-5} benadryl.

The effects of these substances were also studied on the isolated phrenic-diaphragm preparation of the rat (Bülbring, 1946). The technique followed was the same as described in the previous paper (Dutta, 1949). The action of antistin is shown in Fig. 5. In a concentration 4×10^{-5}, antistin increased the height of contractions elicited by single, maximal nerve stimulation by about 20 per cent (Fig. 5*a*). After the preparation had been washed out, antistin produced the same effect when direct stimulation was applied (Fig. 5*b*). After the preparation had been washed out again, the addition of 100 µg. of *d*-tubocurarine chloride to the bath

abolished the response of the muscle to nerve stimulation (Fig. 5*c*) but not to direct stimulation (Fig. 5*d*); the addition of the same amount of antistin to the bath was followed by increased muscle twitches as before. Before the wash-out (W), the stimulation of the nerve had no effect. The contractions began to return some time after the Tyrode solution had been changed again (W). This is shown on the right-hand side of the record (Fig. 5*d*). In higher concentrations antistin caused a curare-like depression of the rat diaphragm. The minimum concentration necessary to demonstrate this varied with different preparations. In the experiments illustrated in Fig. 6B, the effect of antistin in a concentration 1.6×10^{-4} was most pronounced. Depression of the muscle was observed with only one quarter of this concentration in other experiments. When the concentration of antistin was high enough to cause marked diminution of the twitch tension of the rat diaphragm, the initial stimulatory stage was seldom missed. However, this was barely evident in the experiment reproduced in Fig. 6B.

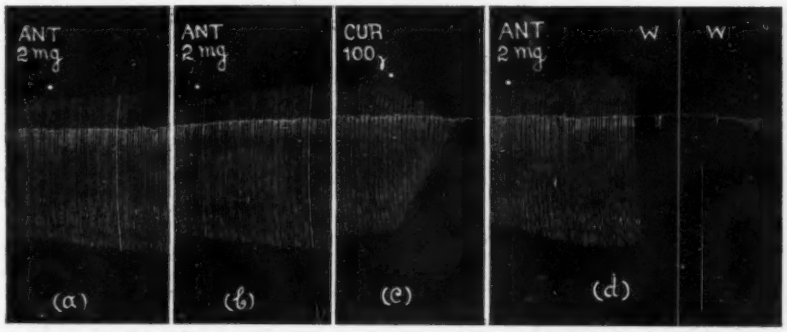

FIG. 5.—Rat diaphragm phrenic nerve preparation in Tyrode solution, 37° C., 50 ml. bath. Single, maximal shock, 7 per min., 0.7 millisecond. Tyrode solution changed between (*a*) and (*b*) and between (*b*) and (*c*). (*a*) Indirect stimulation: 2 mg. antistin increased the muscle contractions. (*b*) Direct stimulation: 2 mg. antistin had the same effect as in (*a*). (*c*) Indirect stimulation: progressive paralysis of the muscle to nerve stimulation after 100 μg. *d*-tubocurarine chloride. (*d*) Direct stimulation: 2 mg. antistin still enhanced the contractions. Before being washed out (W) the muscle gave no response to indirect stimulation, but the contractions began to return after the second washing out, as shown in the right-hand side of the record.

The action of benadryl was very similar to antistin. This substance in a concentration 4×10^{-5} augmented the contractions of the rat diaphragm by 78 per cent on indirect stimulation and by 50 per cent when the muscle was stimulated directly. After the diaphragm had been made insensitive to nerve stimulation by the addition of 100 μg. of *d*-tubocurarine chloride to the bath, the same concentration of benadryl still increased the muscle twitches in response to direct stimulation by 23 per cent. In these experiments the rate of stimulation was 7 per min., the duration of each stimulus being 0.7 millisec. Benadryl in a concentration 4×10^{-5} also caused depression of the contractions in response to nerve stimulation. This decline in the height of muscle twitches was usually preceded by a period of augmentation (Fig. 6D).

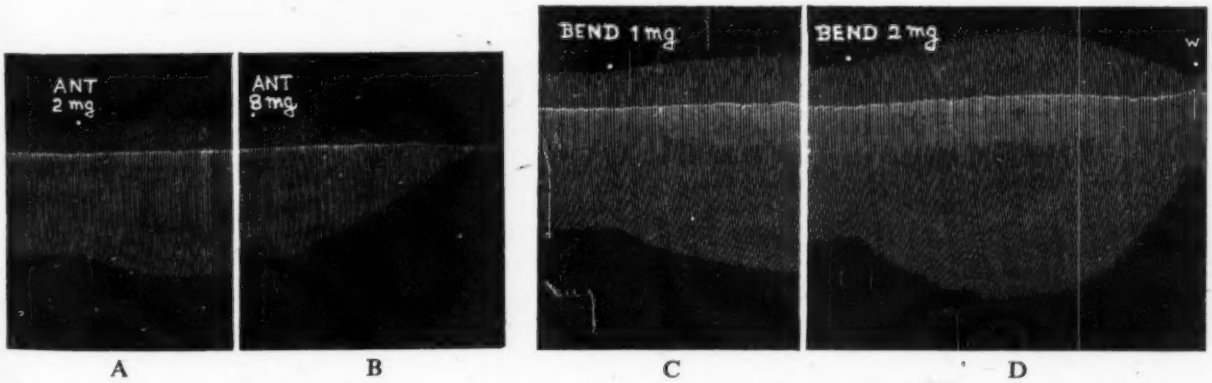

FIG. 6.—Rat diaphragm phrenic nerve preparation in Tyrode solution, 37° C., 50 ml. bath. A and B: single, maximal nerve stimulation, 7 per min., 0.5 millisecond. The solution was changed between A and B; 2 mg. antistin increased the contractions (A) while 8 mg. produced depression of the muscle tension (B) preceded by a very short stage of stimulation. C and D: single, maximal nerve stimulation, 6 per min., 1 millisecond. The solution was changed between C and D; 1 mg. benadryl produced an increase in the amplitude whereas 2 mg. caused an initial increase followed by progressive depression till the bath was washed out (W).

Action on the perfused superior cervical ganglion

From the results of the previous experiments a suggestion arose that the curare-like action of these substances on the skeletal muscle might be exerted by blocking the neuromuscular transmission. This led to an examination of the effect of benadryl and antistin on the perfused superior cervical ganglion of the cat. The procedure was the same as described in the earlier paper (Dutta, 1949). The ganglion was perfused with Locke's solution at 35° C. In Fig. 7 (upper record) the depressant action of benadryl is illustrated. The record shows the contractions of the nictitating membrane in response to preganglionic stimulation (16 per sec., every 3 min.). The third stimulation was preceded by a dose of 250 μg. benadryl and the response to the next stimulation was abolished. The ganglion recovered gradually. The effect of antistin is shown in Fig. 7 (lower record) and the results of all the experiments are summarized in Table IV.

TABLE IV

ACTION ON SUPERIOR CERVICAL GANGLION OF THE CAT

Exp. No.	Drug	Amount injected in μg.	Height of contraction of the nictitating membrane in mm.		Percentage inhibition
			Before addition of drug	After addition of drug	
2	Benadryl	100	18	3	83
3	,,	200	65	0	100
4	,,	400	51	37	27
5	,,	400	36	21	42
6	,,	300	30	25	17
7	,,	400	42	34	19
7	,,	600	40	0	100
8	,,	250	47	0	100
4	Antistin	500	40	29	28
5	,,	200	52	48	8
5	,,	400	52	26	50
6	,,	600	28	18	36
8	,,	200	53	39	26

Effect on histamine-induced gastric secretion

There has been some difference of opinion about the effect of benadryl on the gastric secretion evoked by histamine. Experiments were therefore carried out on cats by the method described by Howat and Schofield (1948). A cat, which had been kept without food overnight, was anaesthetized with chloralose and a rubber tube was inserted in the pyloric end of the stomach for withdrawal of the gastric juice. The stomach was washed out with warm water through an opening made in the oesophagus which was reached by an incision in the neck of the animal. This was later closed. The cat was warmed so as to maintain a constant rectal temperature. Atropine sulphate (1 mg./kg.) was injected intravenously to reduce the effect of vagal impulses. After an hour the stomach was washed with N/200 HCl and 25 ml. left in. Every 15 min. the stomach was emptied and refilled with 25 ml. of fresh dilute solution of the acid. The amounts of free and total acid present in each sample were estimated by titration with N/20 NaOH, thymol blue being used as indicator. The samples drawn out immediately before each histamine infusion were taken as the initial level for each secretory curve. Histamine acid phosphate (0.0045 mg./kg./min.) in normal saline was infused intravenously for 45 min. by a slow infusion pump. The total amount of acid secreted during this period constituted the first response. After the secretion had returned to the basal level a second infusion of histamine was given and the total amount of acid secreted formed the second response. Benadryl was injected intravenously before the beginning of the second histamine administration.

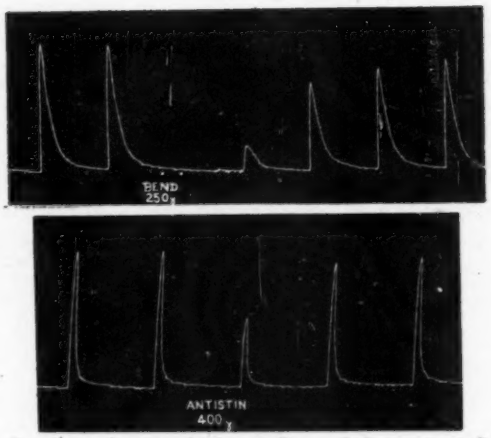

FIG. 7.—Superior cervical ganglion of the cat, perfused with Ringer-Locke's solution. Contractions of the nictitating membrane in response to preganglionic stimuli, 16 per sec. for 10 sec. every 3 min. *Upper record:* response to stimulation was abolished after 250 μg. benadryl. The ganglion recovered shortly. *Lower record:* response to stimulation was diminished after 400 μg. antistin.

The secretion of free hydrochloric acid rose steadily during the infusion of histamine and declined progressively as soon as the infusion ended. It returned to the basal level within an hour. The figures for total and free acid were very close to one another. The amount of acid secreted during each response is expressed throughout as millilitres of

TABLE V

DOUBLE HISTAMINE TEST ON GASTRIC SECRETION IN CATS (AFTER ATROPINE)

1st and 2nd responses = total acid secreted during 1st and 2nd infusions of histamine respectively

Controls				Benadryl 3 mg./kg. between 1st and 2nd responses			
Exp.	Wgt. kg.	1st response N/20 HCl ml.	2nd response N/20 HCl ml.	Exp.	Wgt. kg.	1st response N/20 HCl ml.	2nd response N/20 HCl ml.
1	3.2	10.2	21.1	1	3.2	8.2	13.9
2	3.2	3.3	9.3	2	5.5	18.2	18.1
3	3.3	17.7	11.7	3	3.3	12.0	13.8
4	3.3	10.4	11.4	4	2.8	7.5	31.8
5	2.5	6.5	9.3	5	3.0	7.9	16.0
6	2.5	9.6	22.8	6	3.2	4.8	11.9
7	4.9	21.6	14.1	7	2.8	6.8	18.4
8	4.5	10.3	9.8	8	3.0	8.1	14.1
9	3.4	20.4	18.9	9	2.8	6.1	12.0
10	3.0	12.2	10.0	10	3.0	17.9	10.3
Mean		12.22	13.84	Mean		9.75	16.03
Mean difference 1.6 $\pm$ 2.2, t = 0.73, $\pm$ Std. error p = 0.5, not significant.				Mean difference 6.28 $\pm$ 2.66, t = 2.36, $\pm$ Std. error p is less than 0.05, significant.			

N/20 HCl. When these figures are compared (Table V) the variation between the responses of individual cats becomes obvious. In four of the ten control experiments (Table V), in which no drug was administered between the two injections of histamine, the output of acid during the second response was greater than the first; in another four cats the first response was greater than the second, and in the remaining two no appreciable difference between the two responses was noticed. The mean total amount of acid secreted during the first response (Table V) was 12.2 ml. and during the second response it was 13.8 ml.; the mean difference, which was 1.6 ml., was not statistically significant. Fig. 8 shows graphically the mean results of ten control experiments. In the experiments with benadryl, the mean titratable acidity of the second response was greater than the first by 63.3 per cent (Table V). Since t = 2.36, the value of p was less than 0.05, i.e., the second response differed from the first significantly. Thus after benadryl the gastric cells secreted a greater amount of acid in response to histamine than before the administration of the antihistamine substance.

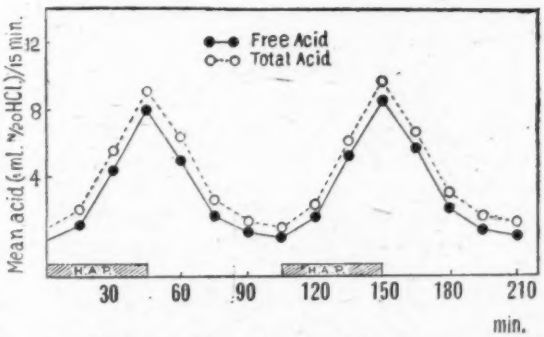

FIG. 8.—Gastric acidity in atropinized cats; double histamine test. Mean of ten control experiments in which no drug was administered between the two histamine responses. Histamine acid phosphate (0.0045 mg./kg./min.) was perfused intravenously for 45 min. each time. The mean second response showed an increase by 13.0 per cent over the first. This was not statistically significant.

DISCUSSION

Benadryl is reputed to possess an atropine-like action, for it reduces the depressor response of acetylcholine in the dog (Loew, MacMillan, and Kaiser, 1946), antagonizes the stimulant effect of acetylcholine on the gut (Loew, MacMillan, and Kaiser, 1946), causes mydriasis, and alters accommodation (Harris, McGavack, and Elias, 1946). Antistin also prevents the contractions of the isolated intestine induced by acetylcholine (Meier and Bucher, 1946). The effect of benadryl and antistin in inhibiting the action of acetylcholine on the isolated rabbit auricles is additional evidence in this direction.

Dawes (1946) reported that the substances which prolong the refractory period of cardiac muscle are antagonists of acetylcholine. This is also true for benadryl and antistin, for both of them reduced the

maximal rate of the electrically stimulated rabbit auricles. This effect is not due to the generalized depression of the auricular tissue. If doses of benadryl and antistin which are sufficient to reduce the maximal rate of contractions of electrically driven auricles are added to a bath in which isolated rabbit auricles are beating freely, the amplitude of contractions is increased. It is necessary to raise the concentration several times before any reduction of the height of contractions or slowing of the organ is noticeable.

Acetylcholine increases the maximal rate of contraction of the driven auricles (Dawes, 1946; Elío, 1947); this effect is increased by eserine or physostigmine and abolished by atropine (Elío, 1947). Elío has further suggested that " the transmission of the impulse in cardiac muscle may be effected by a mechanism in which acetylcholine is a key substance." Recently, Bülbring and Burn (1949) showed that the isolated rabbit's auricle is capable of synthesizing acetylcholine, and that the contractions of an auricle, which has been allowed to beat continuously until it has stopped, can be started again by the addition of acetylcholine to the bath. If, therefore, one assumes that acetylcholine takes part in transmission of cardiac impulses, it is not difficult to explain the action of benadryl and antistin in reducing the maximal rate of the rabbit's auricle; for both of them share the property of opposing the action of acetylcholine at various sites.

On the skeletal muscle of the rat diaphragm these substances produced a dual effect. In small concentrations they increased the response to a single maximal electrical shock, but in higher doses the opposite effect is produced. The evidence is strong that both benadryl and antistin act on the muscle directly, since the augmentation of the muscular twitches caused by indirect stimulation was also seen in response to direct stimulation and was not affected by *d*-tubocurarine chloride. Both benadryl and antistin prevented the contractions of the nictitating membrane in response to preganglionic sympathetic stimulation in the perfused ganglion. It may be that the diminution of the twitch tension of the rat diaphragm in response to nerve stimulation when under the influence of these substances is at least in part due to some blocking of the neuromuscular transmission.

In three of their four dogs with vagal denervated gastric pouches, Loew, MacMillan, and Kaiser (1946) noticed a significant decrease of the acid secretion in response to histamine after benadryl. Using about three times the dose employed by the previous workers, Friesen, Baronofsky, and Wangensteen (1946) failed to observe any such effect. Sangster, Grossman, and Ivy (1947) also did not notice any evidence which justified the conclusion that benadryl diminished the response of the gastric glands to histamine in dogs. Studies on man (Moersch, Rivers, and Morlock, 1946; McElin and Horton, 1946) did not provide any definite evidence that benadryl antagonized the action of histamine on the gastric secretory response. On the contrary, in some subjects the output of acid was actually stimulated (Gilg, 1948; Ashford, Heller, and Smart, 1949). The results of the present investigation clearly show that after benadryl the secretion of titratable acid from the stomachs of atropinized cats is usually increased in response to histamine. Recently Bain, Broadbent, and Warin (1949) have shown that the early effect of the antihistamine substance phenergan is to potentiate the reaction to an intradermal injection of histamine.

Summary

1. Both antistin and benadryl

(i) when tested by the intracutaneous weal method in the guinea-pig, show a linear relationship between the logarithmic concentration of the substances and their local anaesthetic effect. Both are more potent than procaine;

(ii) reduce the depressant action of acetylcholine on the isolated rabbit's auricle;

(iii) decrease the maximal rate at which the isolated rabbit's auricle will respond to electrical stimulation. The relation between the percentage decrease in the maximal rate and the logarithm of the concentration is linear for both these substances. Both of them are more active than quinidine;

(iv) reduce the contractions produced by acetylcholine on the isolated frog's rectus;

(v) in small concentrations increase the twitch tension of the rat's diaphragm elicited by single, maximal nerve volleys. These enhanced muscular contractions are also seen in response to direct stimulation in the curarized preparation. When concentrations of these substances are raised, a decline in the muscle's response to indirect stimulation follows;

(vi) depress the contraction of the nictitating membrane in response to preganglionic stimulation when injected into the fluid perfusing the superior cervical ganglion.

2. In cats after atropine, benadryl significantly increased the rate of hydrochloric acid secretion from the stomach during intravenous perfusion of histamine.

express my grateful thanks to Professor J. H. Burn for his advice, encouragement, and help. I wish to thank Dr. J. W. Trevan, Director of the Wellcome Physiological Research Laboratories, for treating the results on gastric secretion statistically and to Dr. D. Hutcheon for his help.

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THE PRESENCE OF ACETYLCHOLINE IN *TRYPANOSOMA RHODESIENSE* AND ITS ABSENCE FROM *PLASMODIUM GALLINACEUM*

BY

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The finding in this laboratory (Burn and Vane, 1948) that acetylcholine is capable of restoring the spontaneous contractions of isolated rabbit auricles after they have been stopped by the antimalarial substance proguanil raised the question of the possible importance of acetylcholine for pathogenic protozoa in general. The further demonstration of a close relationship between the motor activity of auricular muscle and its ability to synthesize acetylcholine (Bülbring and Burn, 1949) suggested the possibility that this substance might be of particular importance to highly motile organisms such as trypanosomes.

I. Acetylcholine in *Trypanosoma Rhodesiense*

(*a*) *Identification of acetylcholine*

The trypanosome used was an old strain of *T. rhodesiense* which has been maintained at the Liverpool School of Tropical Medicine for the past 26 years. It causes a fulminating septicaemic type of infection in mice and rats, ending fatally three or four days after a light inoculation.

Preliminary experiments confirmed that washed rat blood cells neither contain, nor synthesize under the most favourable conditions, any measurable amount of acetylcholine.

For these experiments rats were bled into 1 per cent (w/v) sodium citrate in normal saline solution containing 1 : 10,000 eserine, the blood cells then being centrifuged, resuspended in eserine-saline, and again centrifuged. The cells thus packed were present in concentrations ranging, in individual experiments, up to 17,800,000 per cu.mm. A mixture was made of equal parts of this packed blood cell material and a substance described by Feldberg and Mann (1946) as "activator," prepared by extracting an acetone-dried powder of rabbit brain with hot saline, and found by these workers to enhance the synthesis of acetylcholine by brain tissue. The mixture was then divided into three portions of 2 c.c. each. One of these was acidified with 1 c.c. N/3 HCl and boiled in order to destroy any possible choline acetylase present; the second portion received no preliminary treatment; while the third was converted into an acetone-dried powder by washing several times in acetone and drying in a vacuum desiccator, the powder being then suspended in 2 c.c. of "activator." The three specimens (2 c.c. each) were next incubated for 75 min. at 37° C. with the ingredients (and in the proportions) shown by Feldberg and Mann (1946) to be suitable for the synthesis of acetylcholine by brain tissue and used also by Bülbring and Burn (1949) for acetylcholine synthesis by rabbit auricles. The specimens, duly neutralized by the addition of N/3 NaOH, were then examined for acetylcholine by their effect on the frog rectus muscle. Any acetylcholine which might be shown to be present in the first specimen (acidified and boiled before incubation) must necessarily have been present from the beginning; any difference in content between the first and second specimens must represent synthesis that had taken place during the period of incubation; whilst acetylcholine shown to be present in the third specimen (the acetone-dried powder) must also have been synthesized during incubation. No acetylcholine could, however, be demonstrated in any of the specimens. Since these tests showed that rat blood cells neither contain nor synthesize acetylcholine, it was accordingly held to be unnecessary to separate blood cells from trypanosomes when preparing a suspension of trypanosomes for investigation of their possible acetylcholine content. The trypanosome material was therefore prepared in the following way.

Six rats were inoculated intraperitoneally (0.3 c.c. per rat) with heart's blood obtained from 2 very heavily infected mice. Two days later the blood of these rats, in turn, was swarming with parasites, and 36 c.c. were obtained by heart puncture and added to 108 c.c. of sodium citrate-saline solution (1 g. $Na_3C_6H_5O_7$, $2H_2O$/100 c.c.; 0.85 g. NaCl/100 c.c.). Eserine, 2.88 c.c. of a solution containing 0.5 g. eserine sulphate/100 c.c. was immediately included, giving a concentration of 1 : 10,000 eserine in the trypanosome-blood mixture. This was then centrifuged at 5,500 r.p.m. for 2 min., the supernatant replaced by saline solution containing 1 : 10,000 eserine, the mixture again centrifuged at 5,500 r.p.m. for 2 min., and the supernatant then discarded. The deposit, consisting of densely packed blood cells and trypanosomes, occupied 14.4 c.c. To this were added an equal volume of saline and 7.2 c.c. N/3 HCl, the mixture then being rapidly boiled and cooled, brought

to 72.0 c.c. in frog-Ringer solution, and stored in the ice-box for determination of acetylcholine the following day.

The material was neutralized and then assayed by four separate methods, two depending on motor and two on inhibitor effects characteristic of acetylcholine. The motor tests were on the isolated frog rectus muscle and the isolated guinea-pig ileum, and the inhibitor tests on the perfused isolated frog heart and the blood pressure of the spinal cat.

Results were as follows:

(i) *Contraction of the isolated frog rectus muscle* (see Fig. 1). In order to deal with the possible complication of non-specific contractions produced by the trypanosome material, a portion of the 72 c.c. obtained as above was treated with N/3 NaOH and boiled to destroy any acetylcholine that might be present. After cooling and neutralizing with N/3 HCl, this control specimen (S.BA.) failed to cause any contraction of the rectus muscle. By adding known amounts of acetylcholine to portions of the control specimen, and matching the contractions which these produced against the contractions produced by untreated portions of the test fluid, 1 c.c. of this fluid (S.) was found to correspond to 0.3 μg. acetylcholine.

(ii) *Contraction of the isolated guinea-pig ileum* (see Fig. 2). The contractions produced by 0.1 c.c. of the trypanosome material were found to be equal to those produced by 0.03 μg. acetylcholine, thus agreeing with the result obtained by the frog rectus method above. That it was acetylcholine which was responsible for the contractions produced by the trypanosome material is supported by the fact that the contractions could be prevented by atropine.

(iii) *Perfused isolated frog heart beat* (see Fig. 3). The diminution which 0.05 c.c. of the test specimen produced in the amplitude of the heart beat was the same as that produced by 0.015 μg. acetylcholine, thus agreeing quantitatively with the results obtained by the two motor tests above. Again the effect could be prevented by atropine.

(iv) *Blood pressure of spinal cat* (see Fig. 4). The blood pressure was reduced to the same extent by 1 c.c. of the test specimen as by 0.3 μg. acetylcholine, thus again agreeing quantitatively with the results obtained by the other methods. No fall in blood pressure was produced by the specimen after it had been boiled with alkali (S.BA.). The fall was increased by prior injection of eserine and abolished by atropine.

FIG. 1.—Contractions of the frog rectus muscle. S.BA. = specimen of trypanosome material boiled in alkali, cooled, and neutralized. S. = specimen not so treated. 1.0 c.c. of S. equivalent to 0.3 μg. acetylcholine (ACH).

FIG. 2.—Contractions of guinea-pig ileum. 0.1 c.c. of trypanosome material (S.) equivalent to 0.03 μg. acetylcholine (ACH). Effect abolished by 20 μg. atropine (ATR).

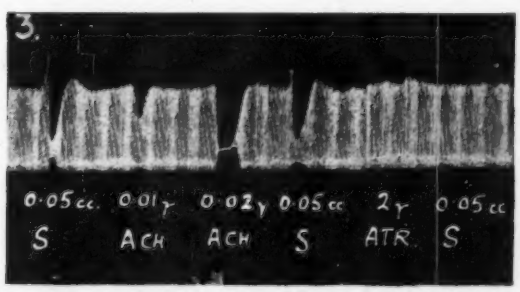

FIG. 3.—Perfused isolated frog heart beat. 0.05 c.c. of trypanosome material (S.) equivalent to 0.015 μg. acetylcholine (ACH). Effect abolished by 2 μg. atropine (ATR).

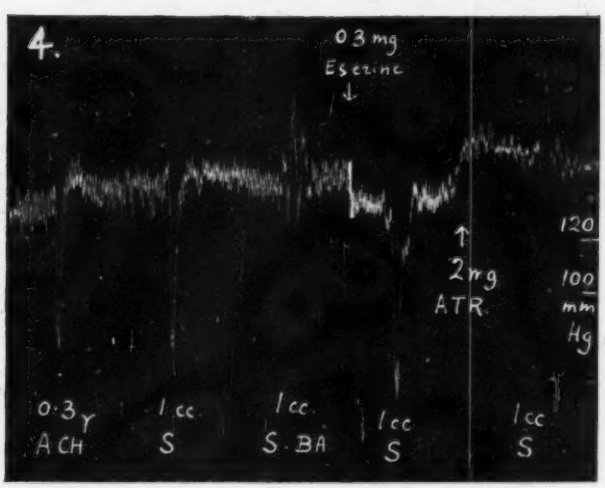

FIG. 4.—Blood pressure of spinal cat. 1.0 c.c. of trypanosome material (S.) equivalent to 0.3 μg. acetylcholine (ACH). No effect produced by 1.0 c.c. trypanosome material previously boiled in alkali, cooled, and neutralized (S.BA.). Effect of 1 c.c. S. enhanced by 0.3 mg. eserine and abolished by 2 mg. atropine (ATR).

The fact that the trypanosome material produced effects characteristic of acetylcholine in each of these four tests, and that, moreover, its acetylcholine content was in perfect agreement (0.3 μg. per c.c.) for each assay method, leaves no room for doubt that it was indeed acetylcholine and not some unidentified substance which was responsible for the reactions observed.

(*b*) *Synthesis of acetylcholine*

The possibility seems remote that the trypanosomes absorb preformed acetylcholine from some organ or body fluid of the host. It would be much more likely that they acquire the substance by their own powers of synthesis, and this we have been able to demonstrate. After a few orientating experiments, the following routine procedure was adopted in the preparation of trypanosome material for this further work.

Four rats were inoculated intraperitoneally with heavily infected mouse blood on which a trypanosome count had previously been carried out, so that the amount injected per rat could be adjusted to contain about 2×10^8 trypanosomes. Two days later the rats were sufficiently heavily infected for the required purpose. It was found advisable to use rats at levels of infection thus obtained rather than rats that are even more densely infected, since trypanosomes obtained from the latter tended to disintegrate soon after bleeding, resulting in an underestimate of the amount of trypanosome material present if there should be any undue delay in counting the trypanosomes. The rats were bled by heart-puncture and 30 c.c. of blood added to 90 c.c. of 1 per cent sodium citrate-saline solution.

The trypanosomes were counted at this stage, and the total of 120 c.c. then divided into two equal portions, to one of which was added 1.2 c.c. 1/200 eserine (final eserine concentration therefore 0.01 per cent). The reason for treating one portion with eserine was to preserve any acetylcholine that might otherwise be destroyed in the course of the subsequent *in vitro* operations. Each of the two 60 c.c. portions, non-eserinized and eserinized respectively (labelled *N* and *E* for convenience), was then divided into 3 equal lots of 20 c.c. each. These were then centrifuged at 5,500 r.p.m. for 2 min., the supernatant discarded and replaced by saline for *N* and by eserine-saline for *E*, centrifuged once more at 5,500 r.p.m. for 2 min., and the supernatant fluid again discarded, thus leaving 3 specimens of *N* and 3 of *E*, each consisting of about 2 c.c. packed blood cells and trypanosomes. The number of trypanosomes in each of the six specimens thus obtained varied in different experiments between 3.04^9 and 6.37^9.

The 3 specimens of *N* and *E* respectively were then treated in essentially the same way as the 3 specimens obtained from uninfected rat blood in the experiment described on p. 290 designed to confirm that the blood cells neither contain nor synthesize acetylcholine; that is, one specimen was acidified with 1 c.c. N/3 HCl and boiled, one received no such preliminary treatment, and one was converted into an acetone-dried powder.

The specimens were then incubated at 37° C. with the ingredients and in the proportions described by Feldberg and Mann (1946) and assays again carried out on the frog rectus preparation. Standard acetylcholine solutions for matching against the samples of unknown content were made up in the same ingredients as those of the latter samples, except that rat blood cells were not always included. Tests showed that the omission of this particular component did not affect the result. Errors due to contraction of the frog rectus by factors other than acetylcholine were eliminated also by boiling a portion of the unknown solution in the presence of N/3 NaOH, neutralizing, and making up with known amounts of acetylcholine for matching against the unknown. Since the blood cells neither contain nor synthesize acetylcholine, any of this substance discovered must have been derived from the trypanosomes, and, again, the difference in acetylcholine content between the specimen acidified and boiled before incubation and the specimen not treated in this way must represent the amount synthesized during the course of incubation. Any acetylcholine present in the specimen prepared from the acetone-dried powder must also represent synthesis.

The accompanying Table shows the results of a number of consecutive experiments of this type. The amounts of acetylcholine have been calculated per 10^{10} trypanosomes because that number of packed trypanosomes weighs approximately 1 g.; the average of three determinations (in good agreement) of the wet weight of trypanosomes separated from blood cells and packed by centrifugation at 5,500 r.p.m. for 3 min. was, in fact, found to be 0.957 g. The variations of the results shown in the Table are due not merely to experimental error but to the fact that findings are included of the initial orientating experiments of the series before the procedure had been finally standardized as described above. Thus, the absence of acetylcholine in the specimen without eserine (*N*) of exp. 5 may have been due to the fact that there was an excessive concentration of trypanosomes in the specimen, resulting in their disintegration and the destruction of any acetylcholine that may have been present. Moreover, in these earlier experiments, considerable time was taken up by alternately freezing and thawing the specimens, a procedure which was discarded as unnecessary in the later experiments of the series. In the last three experiments, the acetylcholine contents of the preparations without eserine (*N*) were no longer significantly lower than those of the preparations with eserine (*E*). The fact that the use of eserine in preparing the specimens for incubation increased the apparent yield of acetylcholine in the earlier experiments affords additional support for

TABLE

ACETYLCHOLINE IN *Trypanosoma rhodesiense*, IN μG. PER 10^{10} TRYPANOSOMES (0.96 G. WET WEIGHT)

Experiment	Trypanosomes not exposed to eserine before incubation (*N*)			Trypanosomes exposed to eserine before incubation (*E*)		
	Content before incubation	Synthesized during incubation		Content before incubation	Synthesized during incubation	
		Trypanosomes not converted to acetone-dried powder	Trypanosomes converted to acetone-dried powder		Trypanosomes not converted to acetone-dried powder	Trypanosomes converted to acetone-dried powder
1*				2.67	0	0.60
2*a*				2.41	0.21	0.90
2*b**				2.28	0	0.28
3	3.58	1.80	0	4.54	0	0
4	1.86	0.94	0.28	5.08	1.58	0
5	0	0.71	0	8.08	1.60	1.15
6	10.3	5.58	1.24	8.23	4.23	0.74
7	2.71	2.41		3.08	2.56	
8	5.72	2.18		6.80	1.36	

* Trypanosomes separated from blood cells before incubation and assay.

believing that it is indeed acetylcholine and not some unknown substance with which we are dealing.

In two of the earlier experiments (Nos. 1 and 2*b*) the red cells had been carefully separated from the trypanosomes by differential centrifugation before incubation and assay of the latter, but the results as a whole appear to show that there is no advantage in this precaution.

Confining our attention now to the eserinized series alone, the mean acetylcholine content before incubation was found to be 4.79 μg. (range 2.28 to 8.23 μg.) per 10^{10} trypanosomes, or 5.00 μg. (range 2.38 to 8.60 μg.) per g. wet weight. Synthesis ranged between 0 and 4.23 μg. per 10^{10} trypanosomes in 75 min. In order to compare these figures for synthesis by trypanosomes with those found by Bülbring and Burn (1949) for rabbit auricles we have determined that an acetone-dried powder of 10^{10} trypanosomes weighs about 0.078 g. The amounts synthesized by trypanosomes were therefore 0 to 54.2 μg. per g. powder (71.5 μg. in one result of series *N*) as compared with 40.2 ± 12.7 μg. quoted by Bülbring and Burn for rabbit auricles. It is interesting that the figures for trypanosomes and for rabbit auricles should be in such close agreement. It might be emphasized, though, that we have not explored the optimum conditions for acetylcholine formation by trypanosomes. We have merely used those which Feldberg and Mann (1946) found to be optimal for brain tissue; under other circumstances, as yet unknown, it may well be that much higher figures could be obtained for the activity of trypanosome material in this respect. The Table shows that no particular advantage was gained by preparing an acetone-dried powder of the trypanosome material, and this procedure was therefore omitted in the last two experiments of the series.

II. LACK OF ACETYLCHOLINE IN *Plasmodium Gallinaceum*

In order to discover whether the blood stages of *Plasmodium gallinaceum* contain or are capable of synthesizing acetylcholine, the procedure adopted was the same as for *Trypanosoma rhodesiense*, except for minor differences necessitated by differences in the two types of infection. For example, in order to arrive at an estimate of the number of parasites in each specimen, trypanosomes were counted directly in a haemacytometer chamber (after suitable dilution of the specimen), whereas for malaria parasites the total number of red cells was first estimated in the usual way in a counting chamber, the number of parasites then being derived from the ratio of parasites to red cells as determined in stained blood films.

Several experiments were carried out with *P. gallinaceum*, the following being a typical example. A massive infection was produced in an eight-week-old chicken by inoculating it intravenously with 10 c.c. blood from another heavily infected chicken. Two days after inoculation 22 c.c. blood was obtained by heart puncture and immediately added to 66 c.c. sodium citrate-saline solution containing 1 : 10,000 eserine. The mixture was then divided

into 3 equal portions, centrifuged, the supernatant replaced by eserine-saline, and the mixture again centrifuged. The three deposits (containing 9.72^9 parasites each) were then treated exactly as described above for the investigation of rat blood and trypanosomes; that is, one specimen was acidified with 1 c.c. N/3 HCl and boiled, one received no such preliminary treatment, and one was converted to an acetone-dried powder. The specimens were then incubated in the manner described above, and tested on the frog rectus preparation. No evidence of acetylcholine could be detected in any of the specimens tested.

Discussion

Since the isolation by Ewins (1914) of acetylcholine from a liquid extract of ergot, implying its production by *Claviceps purpurea*, very little attention has been directed toward the possibilities of acetylcholine formation by other micro-organisms. A recent and convincing contribution in this field has been the demonstration by Stephenson and Rowatt (1947) of acetylcholine production by a strain of *Lactobacillus plantarum* from sauerkraut. Bayer and Wense (1936) showed acetylcholine to be present in a species of the free-living protozoon Paramoecium, but there seems to have been no investigation till now of the production of acetylcholine in pathogenic protozoa.

It is not possible at the present stage to do more than speculate, in seeking for an interpretation of our finding that trypanosomes synthesize and presumably utilize acetylcholine while malaria parasites do not. It may, however, be relevant that acetylcholine is well known to be intimately concerned in a variety of motor mechanisms among higher organisms ranging from some of the invertebrates (e.g., the leech) up to the highest forms of vertebrate life. Its importance for motor processes has recently been suggested even in a location where it was formerly regarded as essentially an inhibitor substance, namely, in heart muscle; for the work of Bülbring and Burn (1949) provided evidence for believing that the activity of auricular muscle is inseparably linked with its synthesis of acetylcholine. One is therefore tempted to correlate the presence of acetylcholine in trypanosomes and its absence from malaria parasites with the fact that the former are highly active motile organisms while the latter are relatively immobile. This reference to the lack of motility on the part of malaria parasites should, of course, be qualified by recognizing, firstly, that it is only the blood stages and not the mosquito phases with which we are here concerned; and, secondly, that slight motility, varying in degree among different species of malaria parasite, is in fact exercised during the trophozoite (or amoeboid) stage of development. Indeed, the specific name *Plasmodium vivax* testifies to the prominence of this particular feature in the parasite of benign tertian malaria, in comparison with other species of malaria parasite. The motility is, however, relatively sluggish, being essentially amoeboid in type and quite unlike that of trypanosomes with their highly specialized organs of locomotion. The demonstration by Bayer and Wense (1936) of acetylcholine in Paramoecium may be significant in this connexion since Paramoecium is, of course, also characterized by morphological differentiation providing for a high degree of motility.

It need hardly be added that we do not attempt to assign to acetylcholine some role in connexion with high motility as its sole possible function among micro-organisms. Its presence in *Claviceps purpurea* and *Lactobacillus plantarum* is alone sufficient to indicate that this cannot be the case. Likewise, we do not exclude the possibility that a high degree of motility may occur in some species of protozoa or related organisms without acetylcholine being involved. Among the lower orders of metazoa Bacq (1947) points out that coelenterates and tunicates, which are far from devoid of motor properties, contain either no acetylcholine or only inappreciable amounts of that substance.

Summary

1. *Trypanosoma rhodesiense* has been found to contain acetylcholine to the extent of 2.28 to 8.23 μg. per 10^{10} trypanosomes, or 2.38 to 8.60 μg. per g. wet weight. The formation of acetylcholine has been demonstrated *in vitro* in amounts up to 5.50 μg. per 10^{10} trypanosomes, or 71.5 μg. per g. acetone-dried powder, in 75 min. at 37° C.

2. *Plasmodium gallinaceum* has been found neither to contain nor to synthesize acetylcholine.

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THE FATE OF TUBOCURARINE IN THE BODY

BY

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The need for isolating a purified active principle from crude curare started at an early date in South America, when Boussingault and Roulin (1828) succeeded in obtaining a bitter principle which they differentiated from strychnine, isolated eight years previously. Although the problem was somewhat clarified by the work of Preyer (1865) and Boehm (1886, 1897), it was not until 1935 that the active alkaloidal salt, *dextro*-tubocurarine chloride, was isolated in a pure crystalline state by King from a sample of native tube-curare. The same alkaloid was obtained in a good yield by Wintersteiner and Dutcher (1943) from a single plant species, *chondrodendron tomentosum*, which is probably its chief botanical source.

The fate of this alkaloid in the body has received little attention; the present work is an attempt towards providing some information on this point.

Choice of a Method

The methods of assaying curare-preparations in general are either chemical or biological.

Chemical methods.—Barbosa (1903) published elaborate charts depicting the colours obtained with various curare compounds. Qualitative, though non-specific, reactions for the curare alkaloids with potassium ferrocyanide were described by Cole (1923) and with trichloracetic acid by Schoofs (1927). Recently, Foster and Turner (1947) have developed a tentative polarimetric method for the assay of *d*-tubocurarine chloride. They also described a colorimetric method for the assay of the alkaloid depending on the use of Folin-Ciocalteu phenol reagent.

All these methods, however, must be of limited application on account of the many materials which yield colours with the reagents, and the high concentration of the alkaloid required for its detection.

Biological methods.—One of the early methods was described by Gaddum (1937) and consisted in determining the paralysing dose for the frog. Holaday (1941) developed the head-drop method, which employs muscular relaxation in an intact mammal (rabbit) as the criterion of curare activity. The intravenous injection of curare in mice likewise produces a head-drop. This method, described by Kimura and Unna (1948), is claimed to be more economical and allows the statistically valid determination of the head-drop dose on a uniform population. In this connexion it may be noted that the difference between the average head-drop dose and the average lethal dose represents the margin of safety of the drug, and that this margin is so narrow that the determination of the average head-drop dose often results in loss of the animal. Moreover in a head-drop method the results are not recorded objectively.

Skinner and Young (1947) described a "mouse-method" of assay of curare activity as being simple and objective. Marsh and Pelletier (1948) used a method depending on comparing the paralytic doses for rats.

The main use of any of these methods at present is mostly confined to the evaluation of compounds with curare-like activity, and even there they suffer from the disadvantage that they do not establish the site of action of the drug, which is one of its most characteristic features.

The isolated frog's nerve-muscle preparations (gastrocnemius-sciatic or nerve-sartorius) have also been used; most authors (for references, see Ing, 1936) have estimated either concentrations which paralysed the muscle completely or which just failed to cause complete paralysis. After such severe poisoning, recovery is usually slow.

Chou (1947) used the rat's phrenic nerve-diaphragm preparation (Bülbring, 1946) for the estimation of curare-like activity. The use of this method for assay requires the presence of the test substance in relatively large concentrations.

The frog's rectus abdominus muscle method

This method depends on a measurement of the antagonism between acetylcholine and tubocurarine, and although it is probably more sensitive than other

methods it is not specific. It was described by Jalon (1947) and was found suitable for the purpose of the present work. The bath used here for this preparation contains 2 ml. Ringer's solution at room temperature, aerated by a continuous stream of oxygen bubbles, and the contractions are recorded on a slowly moving smoked drum. The magnification is about 10 and the tension about 3 g. weight. A suitable fixed dose of acetylcholine is added to the bath every 6 min. and allowed to act for exactly 2 min. before it is changed and the preparation allowed to relax. When the responses of the rectus muscle to acetylcholine have become quite regular, a suitable volume of the solution to be tested is added 90 sec. before the addition of acetylcholine, and its effect on subsequent responses to the latter observed. When doses are thus added at a constant time interval, the effect produced in the given constant time is regularly related to the dose and can be taken as an index of the potency of the solution. Thus a quantitative estimate of the amount of tubocurarine present in an extract is obtained by comparing it with a standard solution of tubocurarine, given in alternate doses. By this procedure it is possible to detect 0.1 μg. tubocurarine. As it made no difference to the assay whether or not the rectus had been sensitized beforehand by eserine, the uneserinized preparation was preferred.

The Preparation of Extracts

In extracting added or injected tubocurarine from the various tissues and biological fluids, the following methods were tried:

Blood.—Using whole blood *in vitro*, most of the drug added was recovered from the plasma by the use of acid alcohol. Extracts of blood cells gave no evidence that tubocurarine had passed inside them. After an injection, extracts were made in the following way: The blood was mixed with heparin and cooled with ice, and the plasma immediately separated. The acid alcohol was prepared by acidifying absolute ethyl alcohol with a crystal of tartaric acid or with 0.1 ml. *N*.HCl, and 10–15 ml. were used for each 1 ml. plasma. The plasma was added dropwise to the acidified alcohol, which was shaken thoroughly after each addition. The precipitate was separated and washed with acid alcohol, the alcoholic solution taken down to dryness on a water bath, and the residue dissolved in Ringer's solution and filtered.

Urine.—A certain volume of the urine was evaporated to dryness on the water bath. The solid residue was then thoroughly mixed with absolute ethyl alcohol (5 ml. for each 1 ml. urine) and the precipitate separated by centrifuging and washed with absolute alcohol. The alcoholic solution was then evaporated to dryness, and the final residue taken up in Ringer's solution (1–10 ml. for each 10 ml. urine); thus the tubocurarine in the urine could be concentrated 1–10 times.

Tissue.—A convenient method was found to depend on extraction with acid alcohol, and here the use of sulphuric acid as an acidifying agent was usually found to be superior to hydrochloric acid in providing a final clear extract; this was observed by Chang and Gaddum (1933) when they were estimating the acetylcholine equivalent of tissue extracts. Since the acetylcholine in these extracts would interfere with the test for tubocurarine it was inactivated by hydrolysis. Extracts prepared in this way may contain other pharmacologically active substances, and are therefore only suitable for use in a biological test, which is relatively little affected by these substances; this is another advantage of the frog's rectus muscle which is insensitive to most substances in extracts except acetylcholine.

The tissue was weighed, cut up with scissors, and mixed with acidified alcohol (15–20 ml. per g. tissue), where its cutting up and mixing were completed. This acidified alcohol was prepared by adding 1.2 ml. $2N.H_2SO_4$ to each 100 ml. of absolute ethyl alcohol. The deposit was then separated by centrifuging and washed with acid alcohol; the alcoholic solutions were evaporated to dryness and the residue taken up in Ringer's solution and filtered. The extract was then made slightly alkaline and boiled for 1–2 min. to destroy acetylcholine but not tubocurarine; finally it was neutralized and concentrated until 1 ml. corresponded to 1–5 g. tissue.

Faeces.—In some animal experiments it was desired to look for the presence of the drug in the faeces. Here, the masses were powdered and a weighed quantity transferred to a dry clean mortar and thoroughly mixed with absolute ethyl alcohol (10 ml. per g.); the deposit was separated and washed with alcohol, and the alcoholic solutions finally evaporated to dryness and the residue dissolved in Ringer's solution and filtered.

Gastric juice.—In the conscious human subject, excretion of the drug was sought for in the saliva and gastric juice. The fasting gastric juice, aspirated through a Ryle's tube, was well shaken and filtered. The filtrate was heated on the flame and the coagulum separated; the clear fluid was neutralized and used for the test.

Saliva.—To each 9 ml. absolute alcohol, acidified by few drops of dilute HCl, 1 ml. saliva was added drop-by-drop, the alcoholic solution being shaken thoroughly after each addition and for sufficient time at the end. The thin precipitate was separated and washed with acid alcohol. The alcoholic solutions were evaporated to dryness and the residue taken up in Ringer's solution and filtered.

It may also be mentioned that in extracting biological fluids a control sample of the fluid was always obtained before the injection of the drug was made, and was extracted and examined in the same way as the later samples. When the samples obtained after the injection showed measurable curariform activities, this usually decreased as the time interval after which the sample had been obtained became longer, until it faded away approaching the blank control.

Such an activity was assumed to be due to the presence of the drug in the corresponding fluid. As for the tissues, a control extract was prepared from the corresponding

tissue of a control animal. The control biological fluids and tissue extracts thus obtained were devoid of curariform activity. All extracts were, if necessary, made neutral before they were used in the test.

Results

In order to test the accuracy of the methods used, the recovery of known amounts of *d*-tubocurarine chloride added to the various tissues and biological fluids was tried. Table I shows that the recoveries of known amounts added to blood, urine, and saliva were satisfactory. In Table II the recoveries of known amounts added to the various tissues are recorded.

TABLE I

RECOVERY OF TUBOCURARINE ADDED TO BIOLOGICAL FLUIDS

Biological fluid	Tubocurarine concentration: μg./ml.		Per cent loss
	Added	Recovered	
Human blood	2.0	1.80	+10
	1.0	0.90	+10
	1.0	1.0	0
Rabbit's ,,	2.0	1.9	+5
	1.0	0.85	+15
	1.0	1.12	−12
			Mean + 4.6
Human urine	2.0	1.9	+5
	1.0	1.0	0
Rabbit's ,,	2.0	1.90	+5
	1.0	1.15	−15
Rat's ,,	1.0	1.0	0
	1.0	0.90	+10
			Mean + 0.83
Human saliva	2.0	2.10	−5
	1.0	0.85	+15
	1.0	0.85	+15
			Mean + 8.3

TABLE II

RECOVERY OF TUBOCURARINE ADDED TO TISSUES

Tissue	Tubocurarine concentration: μg./g.		Per cent loss
	Added	Recovered	
Minced mouse	1.0	1.10	−10
	1.0	1.05	−5
	0.5	0.45	+10
Rabbit's liver	1.0	1.07	−7
	1.0	0.9	+10
Rabbit's muscle	0.5	0.45	+10
	0.5	0.4	+20
Rabbit's kidney	0.5	0.45	+10
	0.4	0.35	+12.5
			Mean + 5.6

The fate of tubocurarine in man

This was studied in five subjects, one conscious man and four patients undergoing surgical operations and kept under cyclopropane-oxygen anaesthesia. The study was made by determining the blood levels of the drug at various intervals after its intravenous administration, by determining the amount excreted in the urine and in the conscious subject, by detecting and estimating the drug in some other biological fluids: i.e., the saliva, gastric juice, and cerebrospinal fluid. All control samples were collected before the injection, then the drug, tubarine "B.W.," was injected intravenously in a dose of 0.2 mg./kg. The patients were in the second plane of anaesthesia. Blood samples were drawn on the third, fifteenth, and thirtieth minutes after the injection. Urine specimens were collected at hourly intervals after the injection. Furthermore, in the conscious subject, a sample of saliva was collected on the twenty-first minute. The spinal canal was tapped and a sample of cerebrospinal fluid drawn thirty-three minutes after the injection, and ten minutes later a specimen of the fasting gastric juice was aspirated.

In Fig. 1 the average levels of the drug in the blood are presented. From this curve it may be noticed that: (*a*) an average concentration of about 4 μg. per ml. plasma, occurring three minutes after

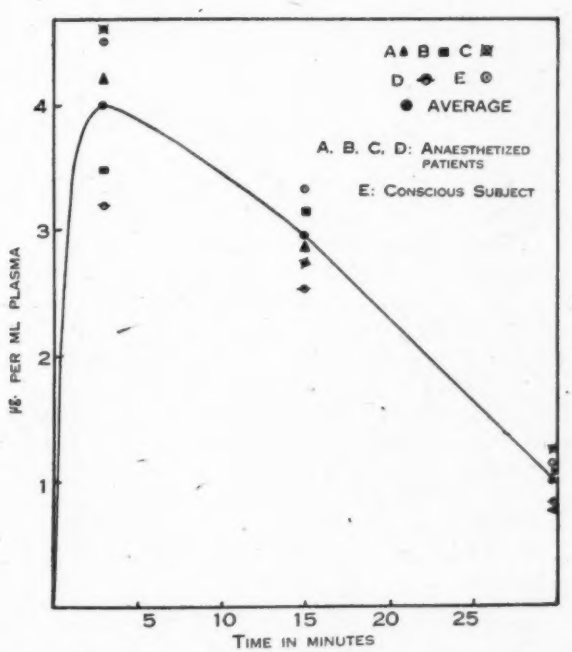

FIG. 1.—Plasma concentrations of tubocurarine in man after the intravenous administration of 0.2 mg./kg.

the injection, seems desirable for the production of full muscular paralysis, providing adequate relaxation for surgical procedures. This corresponds in the conscious subject to the complete classical picture of curarization; (*b*) fifteen minutes after the injection, when the muscles begin to regain their tone, and in the conscious subject their power, the corresponding average concentration is about 2.6 μg. per ml. plasma; and (*c*) half an hour after the injection, when there was apparent recovery from the drug effects, a level of about 1 μg. per ml. plasma was reached.

The various volumes of distribution of the drug at the specified intervals are given in Table III.

TABLE III

VOLUMES OF DISTRIBUTION OF TUBOCURARINE IN MAN

Dose: 0.2 mg./kg. i.v.

Time after dose (min.)	Mean concentration in plasma (mg./l.)	$\text{Log}\left(\frac{\text{mean concen.}}{\text{dose (mg./kg.)}}\right)$	Volume of distribution l./100 kg.
3	4.0	1.30	5
15	2.6	1.11	7.7
30	1.0	0.7	20

In the conscious subject the drug was detected in the saliva in a concentration of 1.2 μg. per ml. twenty-one minutes after the injection; and in the C.S.F. in a concentration of 2.5 μg. per ml. thirty-three minutes after the injection. About 12 per cent of the dose injected was recovered from this subject's gastric juice.

The gastric excretion of tubocurarine was also demonstrated in cats in the following way:

In spinal cats, previously starved for 15–18 hours, the stomach was washed with warm saline solution, tied at the cardia, and filled with 80 ml. saline through a cannula tied into the pylorus. Adequate artificial ventilation was maintained. The drug was injected in a dose of 0.2 mg./kg. intravenously through a cannula connected to the femoral vein. One hour after the injection a sample of saline was withdrawn from the stomach and examined for its tubocurarine content. Two such experiments were performed; in one, the total excretion was equivalent to 19.4 per cent of the dose injected (cat ♂ 2 kg.) and in the other (cat ♀ 2 kg.) to 14 per cent.

Renal excretion.—The tubocurarine equivalents of the hourly samples of urine obtained from the five human subjects are shown in Table IV.

TABLE IV

RENAL EXCRETION OF TUBOCURARINE IN MAN

Dose: 0.2 mg./kg. i.v.

Subject	Dose mg.	Tubocurarine equivalent of urine: mg.					Total excretion as % of dose given
		1st hr.	2nd hr.	3rd hr.	Next 3 hrs.	Total	
A.	14	2.82	1.24	0.50	0	4.56	32.6
B.	15	2.48	1.82	0.72	0.3	5.32	35.5
C.	12	2.20	1.24	0.62	0	4.06	33.8
D.	15	2.6	2.02	1.50	0	6.12	40.8
E.	15	3.0	1.4	1.0	0	5.4	36.0

TABLE V

BLOOD CONCENTRATIONS AND VOLUMES OF DISTRIBUTION OF TUBOCURARINE IN RABBITS

Dose: 0.12 mg./kg. i.v.

Rabbit		Tubocurarine concentration mg./l. of plasma at times stated after dose		
No.	Weight kg.	2 min.	10 min.	15 min.
1	2	2.1	1.2	0.8
2	2.4	2.0	1.7	1.2
3	2	2.3	1.5	1.0
4	2.5	2.4	1.7	1.1
5	2.8	2.2	1.4	1.0
6	2.8	2.2	1.5	0.9
Mean concentration		2.2	1.5	1.0
$\text{Log}\left(\frac{\text{mean concen.}}{\text{dose (mg./kg.)}}\right)$		1.262	1.097	0.92
Volume of distribution l./100 kg.		5.4	8.0	12.0

The fate of tubocurarine in the rabbit

The drug was injected intravenously in a single dose of 0.12 mg./kg. body weight. At the 2nd, 10th, and 15th min. after the injection blood samples were collected and the plasma separated as usual. Urine samples were usually collected by a sterile catheter at the end of the 2nd, 4th, and 7th hours after the injection. All samples were extracted and examined for their curariform activity. Six rabbits of both sexes were used.

In Table V the blood concentrations and volumes of distribution at the stated intervals after the administration of the drug are shown. Fig. 2

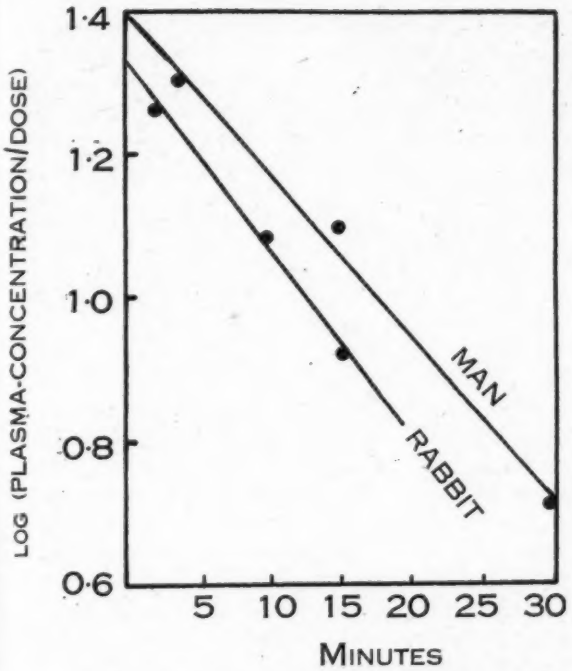

FIG. 2.—The concentration-dose relationship after intravenous administration of tubocurarine (0.12 mg./kg.) in the rabbit and (0.2 mg./kg.) in man.

represents the relationship between time and the logarithm of the ratio: $\left(\frac{\text{mean conc. in plasma}}{\text{dose: mg./kg.}}\right)$ for man and the rabbit. In this Fig. it may be noted that the ordinate corresponding to zero time for man is 1.38 or log 24; the volume of immediate distribution is thus estimated as 100/24 or 4.2 per cent, which is probably about equal to the plasma volume. For the rabbit the volume of immediate distribution calculated in the same way was 4.7 per cent. This is consistent with the finding that the drug does not pass into the blood cells. It disappears from the plasma exponentially, with a halving time of about 13 min.

The average total excretion of the drug in the rabbit's urine was found to be 35 per cent of the dose given; of this about 23 per cent was excreted in the first two hours and 12 per cent in the second two hours. Samples collected at the end of the seventh hour were free from the drug.

Distribution in rabbit's tissues

The distribution of the drug in the rabbit's tissues was examined in two rabbits of different sex and of equal body weight. Ten minutes after the intravenous injection of 0.17 mg./kg. the animals were killed by stunning and bleeding, and their organs removed and extracted. Extracts of brain, kidneys, liver, and voluntary muscles were examined, and the results are shown in Table VI.

TABLE VI

TUBOCURARINE-EQUIVALENTS OF RABBIT'S TISSUE EXTRACTS

Rabbits killed 10 min. after the intravenous administration of 1.17 mg./kg.

	Voluntary muscle	Brain	Kidneys	Liver
Tubocurarine equivalent (μg./g.):	0.16 0.15	0.12 0.15	1.6 2.5	0.10 0.13
Total equivalent of organ (μg.):	144 135	1.27 1.50	25.6 35.0	9.5 11.44
Percentage of dose in organ:	42 40	0.37 0.44	7.5 10.3	2.8 3.4
Average % of dose in organ:	41	0.41	8.9	3.1

In one rabbit the muscle extract was prepared from neck muscles and in the other rabbit from the thigh muscles. No appreciable difference between the concentrations of the drug in the two extracts was noticed. The calculation of the total tubocurarine-equivalent of voluntary muscles was made on the assumption that they constitute 45 per cent of the total body weight.

Oral administration

Rats of both sexes weighing between 200 and 280 g. were used, and tubocurarine was administered by a stomach tube after the animal had been starved over-night. The results are shown in Table VII.

TABLE VII

FATE OF TUBOCURARINE GIVEN BY STOMACH TUBE TO RATS
(No drug detected in faeces)

Dose mg./kg.	Number of rats	Effects	Urine % of dose
10	5	None seen	Nil
25	5	,, ,,	,,
30	2	Variable paralysis	0.15
35	5	,, ,,	0.1
40	3	Severe paralysis	0.2
42	5	Severe paralysis and death	
45	3	do.	—

From these experiments it became clear that the drug is absorbed from the gastro-intestinal tract. In an attempt to localize the site of absorption from this tract the following experiments were performed.

Group I rats.—Each animal in this group was starved over-night and in the morning a median laparotomy incision was made under ether anaesthesia and the duodeno-pyloric junction secured and tied. Then a stomach tube was passed through the mouth, with the animal still under anaesthesia, and tubocurarine injected through the tube into the stomach. The abdominal incision was then stitched up quickly and the animal allowed to recover from the anaesthesia. The presence of large amounts of the drug (50, 100, and 120 mg./kg. body weight) introduced into the stomach in this way was without any obvious effects for a period of two hours, after which the animal was painlessly killed. Two animals were given each of the lower doses and four the higher dose. The weight of these rats ranged between 200 and 260 g. When the animal was killed and the stomach contents examined, practically all the amount introduced was recovered from there; the drug was not absorbed by the gastric mucosa.

Group II rats.—Each animal was starved over-night and in the morning a median abdominal incision made under ether anaesthesia and the duodeno-pyloric junction secured. A stomach tube was passed through the mouth and manipulated from the abdominal wound into the duodenum. The tube was then kept in position by a loose loop placed around the duodeno-pyloric junction. The drug was introduced into the small intestine by injecting it through the stomach tube. Then the latter was carefully withdrawn while the loose loop was tightened around the duodeno-pyloric junction. The laparotomy incision was then quickly stitched up and the animal allowed to recover from the anaesthesia.

On recovery from anaesthesia, these rats were observed to pass quickly into a typical condition of curare paralysis of variable severity. In six rats weighing between 200 and 250 g. when the dose of tubocurarine left inside the intestine was over 3 mg./kg. (3.5 mg./kg. in four rats and 4 mg./kg. in two rats), paralysis was very severe and progressed to complete respiratory arrest and death in 3–8 min. Doses of 2–3 mg. per kg. invariably produced a certain degree of paralysis of variable severity, starting about 4–6 min. after the internal administration. This paralysis extended over a period of 15–25 min. and was severer when the dose left inside the intestine was 3 mg./kg. Four rats weighing between 200 and 260 g. were used for each dose level.

It was also noticed that the duration of action of the drug was rather short, although absorption started fairly soon. It was suspected that the pancreatic juice might be causing inactivation of the drug. In order to investigate this the pancreatic juice of a cat, prepared by the method recommended by Sherrington (1919), was incubated with tubocurarine at 37° C. and the curariform activity of the mixture evaluated at the end of two hours. No loss of the tubocurarine content of the mixture was detected. The pancreatic juice thus does not appear to catalyse the destruction of tubocurarine.

The effect of water diuresis

In these experiments the tubocurarine-equivalent in the urine of a group of rats was determined after the intramuscular administration of 0.3 mg. tubocurarine per kg. These rats were starved over-night, and on the following day they were injected with the same doses of the drug, just after they had received 50 ml. water per kg. body weight by a stomach tube, and the tubocurarine equivalent of their urine was again determined. Three groups of rats, A, B, and C, each containing three male rats, were used. The total weight of group A was 745 g., of B 750 g., and of C 730 g. In all groups urine specimens were collected at the end of the fifth hour and the ninth hour after the injection; the second samples were inactive, but the fifth hour samples showed curariform activity. When the total tubocurarine-equivalents of the urine were calculated, in each case, before and after the water diuresis, an increase in the total equivalent was noticed to have occurred during the water diuresis. These results are shown in Table VIII.

TABLE VIII

URINARY EXCRETION OF TUBOCURARINE IN RATS, WITH AND WITHOUT WATER DIURESIS

Dose: 0.3 mg./kg. intramuscularly

Rats	Urine volume (ml. in 5 hr.)	Total tubocurarine-equivalent excreted (μg.)	% of amount administered
Group A.	5	44.7	20
Group B.	3.8	52.8	23.5
Group C.	4.5	39.4	18
After 50 ml. water/kg. body wt. by stomach tube:			
Group A.	12.5	68.2	30.6
Group B.	15	63	28
Group C.	18	67.8	31

It was also noticed that although the kidneys seem to be an important organ in the elimination of the drug, renal damage did not prevent full recovery of the animal from the paralytic effects of the drug. This was illustrated by a series of experiments on doubly nephrectomized rats, where it was observed that the removal of both kidneys did not seriously affect the reactions of the animals to tubocurarine. Such animals were apparently able to cope with paralytic doses of the drug (0.3 mg./kg. body weight intramuscularly), and, although the average duration of action of the drug was increased by about 30 per cent, the recovery of the animals by the end of this period was almost complete. Hepatectomy (about 75 per cent of the liver) did not appreciably affect the sensitivity of rats to the drug.

TABLE IX

BALANCE SHEET, SHOWING RECOVERY OF TUBOCURARINE IN MICE

Dose: 0.2 mg./kg. i.v.

Time in hours	Wt. of mice (g.)	Dose per mouse (mg.)	Per cent recovery		
			Mice	Excreta	Total
0	20, 20	0.004	92	0	92
	22, 22	0.0044	93	0	93
1	20, 20	0.004	76	0	76
	25, 25	0.005	80	0	80
4	22, 22	0.0044	20	30	50
	25, 25	0.005	10	22	32

A balance sheet for tubocurarine.—This was constructed from experiments on mice, in which the drug was injected intravenously in a dose of 0.2 mg./kg. Pairs of male animals weighing between 20 and 25 g. were used and the excreta were collected at variable intervals after the injection. The animals were killed and minced, and the tubocurarine equivalent of the extracts of the mince and of the excreta determined. Table IX shows the relationship between the amounts in the mice and in the excreta and the percentage recovery of the dose.

Discussion

The various stages of tubocurarine paralysis could be correlated with the concentrations of the drug in the plasma, though the concentrations at the neuromuscular junction must be more intimately related to these effects. The degree of this paralysis varies widely in different individuals (Gray and Halton, 1948). In the conscious human subject no apparent changes in the sensations were observed to follow the injection of tubocurarine; this was also observed by Prescott *et al.* (1946) and by Smith *et al.* (1947), although it has been reported by Whitacre and Fisher (1945) that intocostrin produces general anaesthesia. The presence in the C.S.F. of this subject of curariform activity equivalent to 2.5 μg. tubocurarine per ml. may be of clinical interest. It occurred at a time when the concentration of the drug in the plasma was about 1 μg. per ml. Everett (1948) has shown that when the drug is brought into direct contact with the central nervous system in a sufficient concentration, it is liable to set up convulsions of central origin. The occurrence of violent convulsions after the intravenous administration of tubocurarine in a case of schizophrenia was reported by Morrison (1948); this may have been due to a greater leakage of the drug from the vessels of a pathological central nervous system. The drug is excreted by the salivary glands and gastric mucosa. The excretion of curarine along these channels was reported by Koch (1870), and von Huber (1922) drew attention to this fact. In this respect, tubocurarine is behaving in a similar way to some heavy metals and alkaloids, e.g., morphine. The amounts of tubocurarine excreted this way, however, are insufficient to produce poisoning after reabsorption.

The renal excretion of tubocurarine after an intravenous injection is a relatively slow process. In hourly samples taken from man it was possible to detect it in the urine three hours, and sometimes four hours, after such an administration. This might explain the common observation of the anaesthetist that if he has to give a second dose of tubocurarine during a lengthy operation, he usually requires a smaller dose to produce a full effect; some of the previous dose is probably still in the system. In the rabbit, as early as ten minutes after the intravenous administration of the drug, the apparent tubocurarine content of the kidneys per gramme of tissue weight was already higher than that of other organs, where the drug seemed to be uniformly distributed.

Although the kidneys appeared to be playing an important part in the elimination of the drug, yet the relief from the obvious effects of curarization did not seem to depend entirely upon renal excretion. In rats, although the total removal of both kidneys caused a slight increase in the duration of action of the drug, yet the recovery of the animals by the end of this period was complete. Partial hepatectomy

did not appreciably increase the sensitivity of these animals to tubocurarine. It was also concluded by Rothberger and Winterberg (1905), and later by Polimanti (1914), that the liver plays no part in detoxicating the drug.

These findings agree with recent clinical observations by Wall (1947) that renal and hepatic damage do not necessarily constitute a serious contradiction to the clinical use of the drug.

There must be some other mechanism by which tubocurarine disappears from the body. The experiments on mice show that the drug is inactivated in the body, since 60 per cent of the dose injected disappeared in 4 hours. The site of this inactivation is unknown. It may perhaps occur in voluntary muscle which was found to contain 40 per cent of the dose in the experiments on rabbits.

It is almost a popular belief that curare is ineffective when given by mouth, either because it is not absorbed from the gastro-intestinal tract or because it is destroyed there or because it is excreted as quickly as it is absorbed, so that an effective blood level is not easily reached. Bernard (1857) showed that the drug given by mouth to dogs was not destroyed by the gastric juice.

Here it was noticed that, within a certain range of dosage, typical paralytic effects were produced when tubocurarine was given by stomach tube to rats, thus indicating absorption. However, the presence of large amounts of the drug in the stomach alone (over 100 mg./kg. body weight) was without any obvious effects on the animal; this was probably due to lack of effective absorption from this organ. When the drug was introduced directly into the small intestine, in a much smaller dose (2–3 mg./kg.), signs of absorption developed rather rapidly and progressed fatally with a slight increase of this dose.

It is possible that with such drugs, producing obvious characteristic signs within a short period after administration, the widely different absorbing properties of these neighbouring mucous membranes could be demonstrated pharmacologically. This may be another instance of the use of tubocurarine as a pharmacological tool.

The effects of the drug (2–3 mg./kg.) thus absorbed were, however, of short duration, since in 15–25 min. the animal recovered from the obvious drug effects. It seemed unlikely that the relatively slow renal excretion could be keeping pace with such a rapid absorption to an extent which would prevent the development of a dangerous blood level. It is possible that the continuation of absorption from the small intestine was limited by a process of precipitation and that the drug may be further destroyed along its course in the intestines. Clement and Pistorio (1928) showed that bile and bile salts could precipitate the alkaloid from curare.

The direct administration of the drug into the intestine reduced the size of the effective dose by mouth more than tenfold. It might be possible to imitate this clinically by giving the drug in keratin-coated capsules in the hope of getting desirable effects in spastic paralytic conditions, but probably the limited absorption of the drug from the intestine and the short duration of its action when so absorbed may limit the clinical value of the drug administered that way.

Summary

1. The method described by Jalon for estimating tubocurarine by its action on the frog's rectus abdominis was adopted for determining the drug equivalent of tissue extracts and biological fluids. This method was used to follow the fate of the drug in man and animals.

2. The immediate volume of distribution on intravenous injection corresponds to the plasma volume. The drug does not enter the blood cells. It disappears from the plasma exponentially with a halving time of about 13 min. These conclusions apply both to man and to rabbits.

3. About 20–40 per cent of the drug appears in the urine. This percentage may be increased by water diuresis. Excretion continues for several hours even when the paralysis only lasts about half an hour.

4. The main route of disappearance of the drug from the body does not depend on the kidneys. By extracting whole mice it was shown that about 60 per cent of the dose was inactivated in the body within four hours. The liver is probably not the main site of inactivation. It is possible that inactivation occurs in voluntary muscles, which were found to contain 40 per cent of the dose in an experiment on rabbits.

5. The effective dose by oral administration in rats is about 100 times the effective dose by intramuscular administration. Absorption occurs in the small intestine, but not in the stomach. On intravenous injection, appreciable quantities (12–19 per cent of the dose) may be excreted into the stomach.

It is a pleasure to express my indebtedness to Professor J. H. Gaddum for suggesting this problem, and for continued guidance and stimulating interest. I am deeply grateful to Dr. M. Vogt for the valuable help and criticism she has given throughout this work; and to Dr. J. Gillies and Dr. H. W. Griffith of the Anaesthetic Department, Royal Infirmary, for the facilities they

provided in the collection of samples from patients and for their help in the experiment on the conscious subject. The *d*-tubocurarine chloride used in the animal experiments was kindly supplied by Dr. J. W. Trevan, Director of the Wellcome Physiological Research Laboratories. Part of the expenses of this work was defrayed by the Moray Fund of Edinburgh University.

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THE PRESSOR AND DEPRESSOR EFFECTS OF CERTAIN SYMPATHOMIMETIC AMINES

BY

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Amphetamine and its *dextro*-isomer are widely used in obesity, in patients who are often liable to suffer from hypertension. Methyl-amphetamine (methedrine) and related substances are also frequently given in repeated doses for their blood-pressure raising effect. As the pharmacological action of these agents becomes weaker on repeated administration, it appeared of interest to reinvestigate and compare their effects.

According to Beyer (1946), the diversity of the response to these agents is not adequately accounted for by Gaddum and Kwiatowski's (1938) theory, which explains the action of ephedrine as being due to its power to inhibit the destruction of adrenaline by amine oxidase. The pressor effects of aromatic alkylamines under various experimental conditions were therefore compared with the action of adrenaline and with each other.

METHODS

Cats were anaesthetized with pentobarbitone intravenously or intraperitoneally, or with ether. Blood pressure was recorded by a mercury manometer connected with a cannula in the carotid artery. All drugs were injected into the external jugular vein. Respiration was recorded by means of a tambour attached to the thoracic or abdominal wall.

RESULTS

A slight increase of blood pressure after an injection of ephedrine, racemic or *d*-amphetamine sulphate, or methedrine could be observed with doses of 0.002 mg./kg. and upwards. The rise was usually maximal with 1 mg./kg. In different animals the response varied greatly both in intensity and duration. After 0.2 mg./kg. dexedrine, the pressor effect lasted for eight minutes in one animal, but it took up to fifty minutes in other cats before the original level was reached.

This individual variation was also present in spinal animals. Within the range of pressor responses no definite quantitative difference was observed between the various amines which were tested.

Curtis (1929), in his studies on ephedrine and related substances, has pointed out that the diminished response to repeated injections makes it impossible to compare quantitatively the effect of these substances in the same animal. It was found, however, that some comparison could be made when small amounts of two agents were injected alternately at short intervals. When using this method for racemic amphetamine and its *dextro*-isomer, we observed a gradually diminishing pressor effect as if the same substance had been injected all the time (Fig. 1).

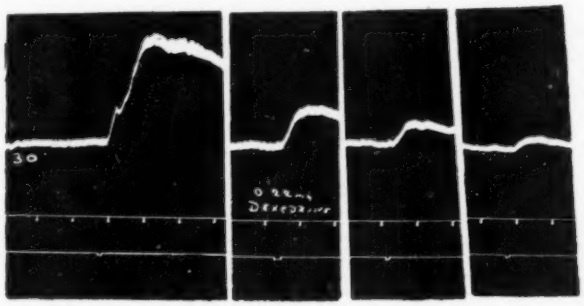

FIG. 1.—Blood pressure effect of alternate doses of 0.1 mg./kg. racemic (A and C) and *dextro*- (B and D) amphetamine sulphate in a spinal cat. Time: minutes.

The small effect of repeated doses of the amines was followed, when injections were continued, by a fall of blood pressure, except with ephedrine, which never caused such an inversion. The amount of the sympathomimetic amine required for the production of such tolerance again varied individually. Usually 1 to 3 mg./kg. were necessary, given either in one or two doses or by injecting smaller amounts repeatedly (Fig. 2). After some hours tolerance to a fresh injection disappeared and

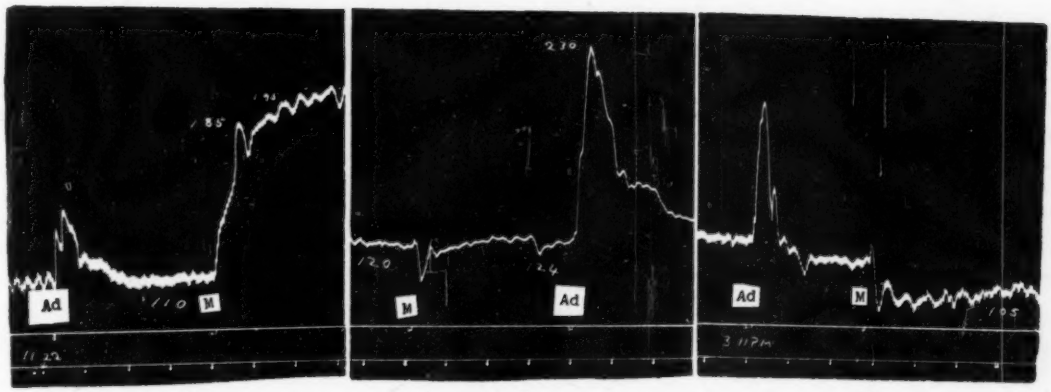

FIG. 2.—Blood pressure effect of repeated doses of 1 mg./kg. methedrine (M) in a cat (pentobarbitone). The pressor effect is marked in A, but reversed in B and C almost 4 hours later; 0.5 ml. adrenaline 1/100,000 (Ad) is pressor throughout. Time: minutes.

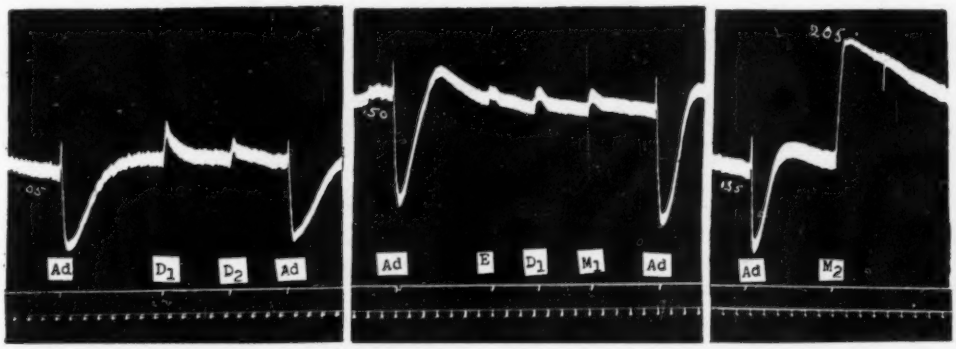

FIG. 3.—Pressor effect of *dextro*-amphetamine sulphate, ephedrine, and methedrine, and depressor effect of adrenaline in a cat (ether). Knee jerk abolished throughout. Time: minutes. Ad = 0.5 ml. adrenaline 1/100,000. D_1 = 0.0017 mg./kg. *d*-amphetamine sulphate. D_2 = 0.00085 mg./kg. *d*-amphetamine sulphate. E = 0.0018 mg./kg. ephedrine. M_1 = 0.0018 mg./kg. methedrine. M_2 = 0.3 mg./kg. methedrine.

a pressor response occurred again. When large doses of the drugs had been given, however, an inverted response was observed even when several hours had been allowed to elapse after the first series of injections. Atropine had no influence on the decreased or inverted responses.

In producing this tolerance to repeated doses the various amines were interchangeable; for example, ephedrine would prevent a rise of blood pressure in response to a later injection of amphetamine or methedrine and *vice versa*. The inverted response after repeated injections was not influenced by atropine, and it was obtained in the decapitated cat.

While the blood pressure effects of ephedrine, racemic amphetamine, dexedrine, and methedrine were very much alike, adrenaline acted differently under our experimental conditions. Under pentobarbitone anaesthesia its pressor action persisted even when large amounts of other amines had been given (Fig. 2).

When, under ether anaesthesia, the response to adrenaline became depressor the other amines retained their pressor effect for their initial doses. This applied both to small and large amounts of the substances (Fig. 3).

The pulse rate in the anaesthetized animals remained substantially unchanged during these depressor responses.

DISCUSSION

Both aliphatic amines and aromatic alkylamines have a decreased blood pressure effect when injected repeatedly (tachyphylaxis). Tainter (1929) has described this for ephedrine, and Ahlquist (1943) has shown it for various aliphatic substances with a sympathomimetic action. Elmes and Jefferson (1942) injected ephedrine into the anaesthetized cat

at hourly intervals and found the pressor effect to decrease gradually; Burn (1946) observed a similar effect with methedrine. Detrick *et al.* (1937), confirming the observation for amphetamine, found in dogs great variability of the pressor response. Pinkston *et al.* (1939) and Pinkston and Pinkston (1939) state that after an initial dose of 1 mg./kg. the magnitude of the response varied directly with the dosage up to 6 to 8 mg./kg. Clinical studies by Myerson *et al.* (1936) and Reifenstein and Davidoff (1938) stress the irregularity of pressor responses to amphetamine in man.

Our experiments show that in the anaesthetized cat 0.002 mg./kg. of these amines will usually produce a very slight rise of blood pressure. The pressor effect becomes sustained after 0.2 to 0.5 mg./kg. and maximal with 1 mg./kg. It may last for almost an hour after a large dose in one cat and for a few minutes only in another. We did not find that the depth of anaesthesia had much influence on this variability.

The strength of pressor action of various agents can be compared in the same animal when small doses are given. Though tachyphylaxis develops, one may find a larger or smaller pressor action when the respective substances are injected at short intervals of one another, but an exact comparison could not be made. Alles (1939) and Hauschild 1940) found that racemic amphetamine and its *dextro*-isomer had the same pressor effect, while Swanson *et al.* (1943) considered the *laevo*-isomer to be more powerful in its pressor action. Our own observations did not show any appreciable difference between the various forms of amphetamine.

That the differences of blood-pressure effects between various animals are not due to a varied sensitivity of the central nervous system and, particularly, that they are not secondary to the action on the respiratory centre was shown in the decapitated cat. Here, as well as when atropine had been given, the same individual differences were observed and tachyphylaxis developed in the same way as in the intact animal.

Several stages could be observed as the animals became gradually saturated with the drugs:

1. The pressor effect became smaller and was eventually absent as if an inert substance had been injected.
2. A momentary drop of blood pressure was immediately followed by a short elevation resulting in a biphasic tracing.
3. The blood pressure fell and returned to its original level sometimes quickly and sometimes very slowly.

However, ephedrine never led to a drop in blood pressure, though the diminution and eventual disappearance of the pressor effect developed as readily as in experiments with amphetamine and methedrine.

The depressor effect was explained by Ahlquist (1943, 1944) and Jackson (1944) as being due to myocardial depression. In flow meter studies on the dog's hind leg, Ahlquist (1945) found that the depressor response was partly due to peripheral vaso-dilatation, and that it was similar to the dilatation after adrenaline. From our observations of the pulse rate during the depressor phase, there was no evidence of a direct effect on the heart. As the depression is not influenced by atropine and as it is also present in the spinal cat, it appears to be due to a vaso-dilator action in the periphery.

The pressor action of aliphatic amines can be blocked by repeating doses or by ephedrine (Ahlquist, 1945). The same applies to the sympathomimetic effect of aromatic alkyl-amines. Hence, once ephedrine, amphetamine, dexedrine, or methedrine have been given in fair doses, further injections will not cause a rise of blood pressure and may, apart from ephedrine, even produce a depressor effect which lasts for a considerable time.

The effect of the amines tested was in our experiments quite different from that of adrenaline. Under pentobarbitone or in the spinal cat the adrenaline response remained pressor. Likewise when under ether anaesthesia adrenaline acted as a depressor, ephedrine, amphetamine, dexedrine and methedrine had their usual pressor effect until tachyphylaxis developed. These differences between adrenaline and the other sympathomimetic amines when given to the same animal at short intervals of each other made it clear that under the given experimental conditions their mechanisms of action are not identical.

It appears difficult to reconcile these results with the theory of Gaddum and Kwiatowski (1938) according to which adrenaline is the substance through which ephedrine acts. If this were true the action of adrenaline and the other sympathomimetic amines would be more or less alike. Tainter (1929, 1933), Aström (1948), and others have shown differences in the effect of ephedrine and other pressor amines.

Tainter (1929) concluded that ephedrine and adrenaline did not have the same seat of pressor action. The opposite responses to the group of sympathomimetic amines on the one hand and to adrenaline on the other shown in our experiments make it likely that their modes of action are different.

Beyer (1946) suggested that the action of sympathomimetic amines might be explained by their effect on the formation of adenosinetriphosphate as influenced by various breakdown products of metabolism and by their influence on the efficiency with which energy is used. This would allow more flexibility than would be permissible if the action of all sympathomimetic amines were mediated through one common agent, adrenaline. It would allow for differences between the effect of adrenaline and the other amines described in this paper. The observation by Govier *et al.* (1945) that certain concentrations of amines lead to an increase of oxidations while larger amounts produce an inhibition of oxygen uptake might help to explain their diminishing pressor effect and the eventual reversal to a depressor action.

We are indebted to Messrs. Menley and James, Ltd., for a supply of dexedrine.

Summary

1. In the anaesthetized and in the spinal cat racemic amphetamine and its *d*-isomer are equally potent in their effect on the blood pressure.

2. Ephedrine differs from amphetamine, its isomers, and methedrine in that on repeated doses it does not cause a fall of blood pressure.

3. As the various amines are interchangeable in blocking the pressor effect an injection of methedrine may result in a drop of blood pressure when either ephedrine, amphetamine, or methedrine itself has been given beforehand in fair dosage.

4. None of these effects is influenced by atropine.

5. The pressor effect of adrenaline persists independently of the changes in the action of the other amines. When, under ether, adrenaline is depressor the other amines have a pressor effect. Under pentobarbitone and in the spinal cat adrenaline still raises the blood pressure when, through repeated injections, ephedrine has lost its effect and amphetamine and methedrine have become depressor agents.

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EFFECT OF REDUCTION OF POTASSIUM ON THE ACTION OF ACETYLCHOLINE ON RABBIT AURICLES

BY

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(Received June 16, 1949)

Spadolini and Domini (1940) were the first to show that minute doses of acetylcholine may stimulate the isolated heart of the guinea-pig and they suggested that this action might be due to a release of adrenaline in cardiac tissue. Hoffmann, Hoffmann, Middleton, and Talesnik (1945) and Haney and Lindgren (1946) have shown that acetylcholine may have a stimulant effect on the heart of the cat or dog after the administration of atropine, and McDowall (1946) has further shown that small doses of acetylcholine may stimulate the cat heart untreated with atropine. Dawes (1946) and de Elío (1947) have shown that acetylcholine reduces the refractory period of the isolated driven auricle of the rabbit, and Wedd and Blair (1946) have demonstrated the direct nature of this effect on nerve-free tissue from the ventricle of the turtle. Recently Burn and Vane (1949) and Bülbring and Burn (1949) have reviewed the literature concerning this subject and as a result of studies of the effect of proguanil and of fatigue on the isolated rabbit auricle have suggested that acetylcholine may play a dual role in the heart. Synthesis of acetylcholine proceeds in the contracting auricle; addition of acetylcholine to produce a concentration above the normal amount present in the tissue causes inhibition of contraction. If the muscle is depressed addition of acetylcholine from without may bring the concentration up to the threshold for normal functioning and bring about stimulation of the heart.

The relationship between the concentration of potassium ions in a tissue and the effects thereon of administered acetylcholine is a close one. In the perfused frog heart increase or decrease of potassium in the perfusing fluid (with appropriate control of the *p*H, saline content, temperature, etc.) reduces the activity of the heart, makes the rhythm irregular and modifies the response to acetylcholine. Similarly increase or decrease of K modifies the contraction of the isolated rabbit auricle.

Experimental

It was decided to test the effect of modification of the ionic content of potassium on the response of the rabbit auricle to added acetylcholine. The beat of the isolated rabbit auricle was recorded as usual at 27° C. in powerfully oxygenated Locke solution with double the normal concentration of glucose. At suitable intervals this fluid was replaced by a modified Locke solution containing half or one and a half the normal amount of potassium. The *p*H was measured and adjusted if necessary by modifying the bicarbonate content to give *p*H 8.2.

The addition of 0.25 μg. acetylcholine in a 75 ml. bath may produce a preliminary stimulation of the auricle of small extent (Fig. 1 *a* and *b*) followed by inhibition. In some specimens this inhibition is not so transient (Fig. 1 *c*) but frequently there is either no effect from addition of such quantities

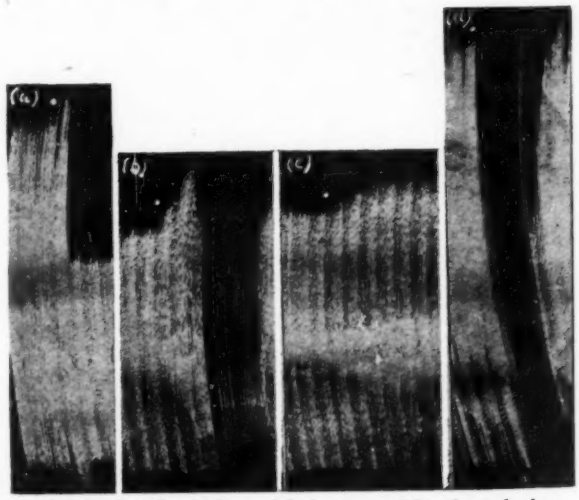

FIG. 1.—Isolated rabbit auricle in normal Locke solution at 27° C. (*a*) and (*b*) show transient stimulation after adding 0.25 μg. acetylcholine to the 75 ml. bath. (*c*) shows a longer stimulation after the same amount of acetylcholine. (*d*) shows inhibition after 1.0 μg.

of acetylcholine or a pure inhibition. The tendency to preliminary transient stimulation develops as the preparation gets older and has been seen in nine out of 47 auricles after two hours beating. In four specimens it was apparent after one hour; it was also seen in two auricles (excluded from the series) which would not beat until they had been treated with 100 μg. acetylcholine followed by washing after a few minutes. Addition of larger quantities of acetylcholine (1 μg. or more) causes inhibition of the activity of these preparations, which may be interrupted by a few beats more powerful than normal (Fig. 1 *d*). Increase of the potassium in the nutrient fluid abolishes this early stimulation with small quantities of acetylcholine, which now have no effect on the heart, or inhibit it.

If the amount of K is reduced the augmentor response to added acetylcholine is enhanced in those preparations which show this effect. Quantities of acetylcholine which, in normal Locke solution, previously or subsequently caused a moderate inhibition of the contraction for a prolonged period might now cause a preliminary stimulation followed by a sharp but transient inhibition, and frequently a prolonged phase of increased activity (Fig. 2 *a*). These effects can be repeated with considerable regularity in those preparations which show the phenomenon (37 out of 45 tests) and are in contrast with the usual action of acetylcholine on the isolated auricle in normal nutrient fluid (Fig. 2 *c* and *d*).

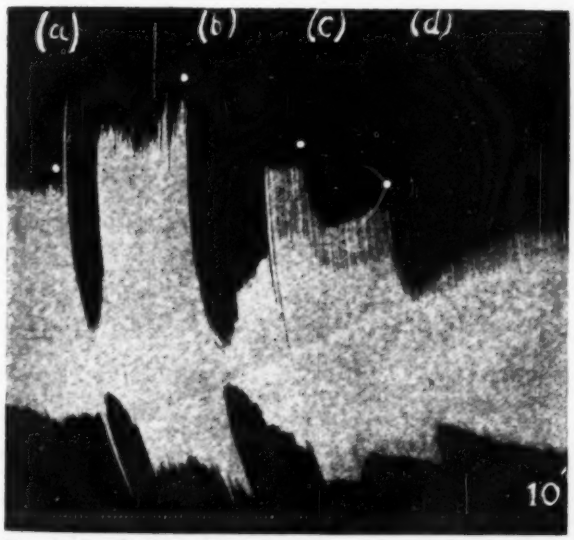

FIG. 2.—Isolated rabbit auricle. (*a*) and (*b*) show the effect of adding 1.0 μg. and 5.0 μg. of acetylcholine respectively, without any wash between, to Locke solution containing half the usual amount of potassium. (*c*) and (*d*) as in (*a*) and (*b*) but in normal Locke solution. For description see text.

DISCUSSION

If isolation of the auricular tissue in Locke solution brings about a state of fatigue and some loss of activity in the muscle, and thus a diminution of synthesis of acetylcholine, addition of a small quantity of acetylcholine will bring the amount of this substance up to the optimal value and cause stimulation of the contraction. This sequence of events is seen in isolated auricles which develop the power of responding to small amounts of added acetylcholine by increased activity, after the preparation has been up for some time. Many specimens never show this property but the same or a similar phenomenon is shown by auricles which have ceased to beat from fatigue and which can be restarted by addition of larger amounts of acetylcholine, with or without washing (Bülbring and Burn, 1949); and by auricles which are inhibited from the beginning, perhaps as a result of rough handling, and which begin to beat after the addition of relatively large doses of acetylcholine, followed by washing out. Reducing the activity of the muscle by increasing the potassium content does not bring about a state of affairs in which acetylcholine causes stimulation. Reduction of the potassium content on the other hand not only modifies the activity of the muscle but also alters the response to acetylcholine, so that amounts of this substance which previously caused inhibition now cause stimulation. Rapid alterations of the response between stimulation and depression may be seen (Fig. 2 *a*) at certain concentration levels of acetylcholine, whereas at higher levels pure depression is observed (Fig. 2 *b*). It may be that an optimum level of potassium is necessary for optimal synthesis of acetylcholine, or for the inhibitory response of the muscle to it. The nature of the response to added acetylcholine would then depend on the condition of the muscle, especially as to its potassium content, and on the amounts of acetylcholine produced in the muscle and applied to it externally, as Burn and Vane suggest. Potassium would appear to play as intimate a role in the positive as in the negative response of cardiac tissue to acetylcholine. According to Jequiér, Plotka, and Petergalvi (1948) the connexion between K and acetylcholine synthesis may lie in the relation between K and degradation of glucose.

SUMMARY

Low concentrations of acetylcholine (3×10^{-9}) may cause a transient stimulation of the isolated rabbit auricle contracting in Locke solution. Higher concentrations inhibit it.

Relative lack of potassium causes doses of acetylcholine, which previously caused a slight transient stimulation, to cause a much larger preliminary stimulation, and some doses which previously inhibited, to stimulate.

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*ISO*PROPYL*NOR*ADRENALINE INHALATION AND MUCOUS MEMBRANES

BY

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Inhalation of aerosols is now widely used in the treatment of bronchial asthma. Numerous substances are employed, of which adrenaline and its related compounds are the most powerful spasmolytics. The relief afforded is so striking (though often temporary) that numerous preparations and nebulizers are bought by the public, very often without medical advice or supervision.

In these circumstances it appears important to know whether inhalation of these compounds may be harmful to the mucous membranes of the air passages. Galgiani *et al.* (1939) found that inhalation of 1 per cent adrenaline in rabbits and cats caused damage to the mucous membranes of trachea and bronchi. Their results are partly supported by earlier findings of Fox (1931), who sprayed the nasal membranes of rabbits with 1/1000 adrenaline and found a mucopurulent discharge.

Very recently *iso*propyl*nor*adrenaline* (aleudrine) has become available. This substance is easily absorbed from the mucous membranes of mouth, pharynx, and the other air passages, and its spasmolytic action appears to be very strong (Herxheimer, 1948) and more powerful than that of adrenaline (Konzett, 1940). Our paper does not deal with the therapeutic merits of *iso*propyl*nor*adrenaline, but as its widespread use can be anticipated it seemed of importance to investigate whether such damage as has been stated to occur after the use of adrenaline would be caused also by this substance.

METHOD

Twelve rabbits were used. They inhaled an aerosol which was produced by connecting one of the commercial glass inhalers to a compressed air cylinder. A steady pressure was maintained from this cylinder, which caused a fine aerosol cloud to flow continuously from the nozzle of the inhaler. Over this nozzle a closely fitting plastic mask was fixed which was pressed over the rabbit's head; the aerosol jet issuing from the nozzle was directed straight against mouth and nostrils of the rabbit and filled the mask, escaping at its upper end. Mouth and nose of the rabbit were thus completely and continuously surrounded by the aerosol. That it actually breathed the aerosol could be seen from the fact that the vapour escaped from the mask in the breathing rhythm of the animal. When the animal breathed out the cloud increased strongly, and decreased during inspiration.

The concentration of *iso*propyl*nor*adrenaline used by one of us (H. H.) for treatment of asthma is usually 1 per cent, if it is nebulized by pressing the rubber ball of a hand inhaler. If a mechanical device, like an air-pressure pump or compressed air cylinder, is used, as little as 0.25 per cent is effective, as much more inhalant is breathed than with a hand inhaler. For this reason we started our experiments with a 0.25 per cent solution, nebulized by compressed air, which was inhaled for 10 minutes every day. The twelve rabbits were divided into three groups of four. One group inhaled pure aleudrine, one group aleudrine to which 0.2 per cent sodium metabisulphite had been added as a stabilizer, and the third group inhaled physiological saline. In each group one drop of glycerine was added to 2–3 c.c. of the solution to delay evaporation and to make the aerosol easily visible.

All the rabbits were treated in this way for 30 days; then one rabbit of each group was killed by a blow on the head. The remainder continued as before for a further 5 days. Then another rabbit of each group was killed. The remaining rabbits were treated with 0.5 per cent aleudrine (or saline) for 22 days. Then a third rabbit of each group was killed. Only one rabbit of each group now remained. They were subjected for 16 days to 1 per cent aleudrine aerosol except the rabbit in the control (saline) group.

When the rabbits were killed, the trachea was quickly excised, opened longitudinally, put in a bath of Ringer-Locke at 39° C., and pinned without stretching on to a cork. A minute drop of india ink was then dropped on to the mucous membrane half an inch below the cricoid cartilage and its movement towards the larynx (due to the action of the cilia) was watched. The speed of the movement over 0.5 cm. was measured with a stop-

* This substance was called "*iso*propyladrenaline" by Konzett (1940), but since it does not contain the N-methyl group of adrenaline it should be called *iso*propyl*nor*adrenaline.—*Editors.*

watch. This method of estimating the action of the cilia described by Hill (1928) did not prove very reliable. Sometimes movement was very strong in one part of the trachea but not in another; sometimes it was so weak that the india ink travelled only 2 or 3 mm. In many cases, however, a speed of about 1 cm. per 50–70 sec. was seen, and there were no differences between the three groups. Weak movement was observed also in the control group.

Trachea and lungs were then fixed in 10 per cent formol-saline and prepared for histological examination, after embedding in paraffin wax. Sections were stained with Ehrlich's Acid Haematoxylin and Eosin, with Heidenhain's Iron Haematoxylin, and with Mayer's mucicarmine stain.

Results

The result of the experiments with ciliary movement has already been mentioned. All the rabbits showed steady gain in weight throughout the experimental period. Histological examination showed no difference between the three groups. The ciliated epithelium was normal. There was no destruction or metaplasia of ciliated epithelium in trachea or bronchi and no polymorphonuclear infiltration of epithelium or submucosa. The goblet cells, stained by mucicarmine, were found in approximately similar density in all three groups. As compared with three rabbits not exposed to the inhaler, mucous activity in the trachea of the experimental animals was slightly increased.

Discussion

There is a striking contrast between our negative results and the positive results of Galgiani *et al.* with adrenaline, applied twice daily by pressing a hand inhaler 10 times. They used therefore an amount of inhalant considerably smaller than that used in our experiments. During one period of inhalation (10 squeezes) they removed 8 mg. of fluid from the inhaler, whereas in our experiments of 10 minutes duration between 600 and 800 mg. were aerolized, the variations being caused by the slight difference in the syphon of the inhaler and of the pressure used. This does not mean, of course, that this amount has been inhaled by the animals. Only a small fraction of it can have been inhaled and still less absorbed.

Of Galgiani's animals (rabbits and cats) exposed to the vapour, 4 died prematurely after 6 to 34 days. Two of these showed inflammatory changes in the respiratory tract. Another animal died on the 117th day of pneumonia and empyema. Five remaining animals were killed after varying periods of time. Two of them showed loss of cilia and three showed mucopus in the bronchi without histological changes. Of the 4 control animals two showed mucopus in the bronchi and a third died with evidence of bronchopneumonia and bronchitis. It must therefore remain doubtful whether this result has sufficient weight to prove a harmful action of adrenaline or of the sodium bisulphite which Galgiani *et al.* used as stabilizer. In our experiments, sodium metabisulphite did not produce any changes.

Our experiments show that aleudrine in therapeutic concentrations is not harmful to the mucosa of rabbits. Whether adrenaline produces harmful results remains an open question.

We are indebted to Mr. T. West for assistance in handling the animals and for the histological preparations.

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THE AMOUNT OF NICOTINE ABSORBED IN SMOKING

BY

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The work which has been carried out in this laboratory on the effect of smoking during diuresis (Burn, Truelove, and Burn, 1945; Walker, 1949) has made it necessary to determine the amount of nicotine absorbed in smoking cigarettes. A few estimates have been published by different workers. These estimates have been made by chemical methods, and the figures vary. Bogen (1929) stated that the amount was between 0.2 mg. and 6.5 mg. per cigarette, according to the rate at which the cigarette was smoked. Schnedorf and Ivy (1939) also give the figure 0.2 mg., but Sollmann (1948) states that under average conditions the amount of nicotine entering the mouth from a cigarette ranges from 1.8 to 8.5 mg., and the amount absorbed ranges from 1.4 mg. to 3.3 mg.

Storm van Leeuwen (1918) estimated the amount of nicotine in cigar smoke, using the blood pressure of the spinal cat. He obtained figures from 1.6 to 4.25 mg. per g. of cigar or 8.0 to 20 mg. for an average cigar.

It is true that cigarettes can be smoked at very different rates, and it is likely that the amount of nicotine entering the mouth will vary considerably according to the rate. In practice, however, cigarettes are smoked at a rate which is about the same for the majority of individuals, and it should be possible to determine the nicotine within narrower limits than those just given.

The work described in this paper consists of two independent investigations, one carried out by H. W. L. in 1935, and the other by C. B. W. P. in 1948, each using the same general method. Since the results of the two investigations are very similar, they are probably a better guide to the amount of nicotine entering the mouth in smoking than are the results given in earlier work. The nicotine was estimated biologically on the blood pressure of the spinal cat.

Ordinary brands of cigarettes were used in both investigations, and a few estimations of cigar and pipe tobacco were also made by C. B. W. P.

METHOD

The cigarettes, cigars, or pipe were fixed in a holder attached to one side of a long glass U-tube, each arm of which was 50 cm. long and of internal diameter 1 cm. The tube was immersed in a large jar of cold running water, and the other arm was attached to a 500 c.c. filter flask, which in turn was attached to a weak suction pump. A T-piece was inserted between the flask and the pump, and when the arm of the T-piece was open, the pump sucked air in through this open arm; when the arm of the T-piece was closed, air was sucked in through the U-tube. A weighed cigarette was inserted in the holder, and when the arm of the T-piece was closed it was easy to light the cigarette. When the U-tube was full of smoke the side arm was opened and suction ceased. The cigarette continued to burn gently in the air as it would if a person were holding it. Aspirations were then continued to simulate as nearly as possible the smoking of a cigarette, that is to say taking about 10 minutes to smoke the cigarette, so as to leave a stub of about 1 cm. The smoke condensed for the most part in the U-tube, any small excess being trapped in the filter flask.

To prepare each extract of cigarette smoke, 5 cigarettes (about 5 g. of tobacco) were smoked in succession in the apparatus. An extract of cigar smoke was obtained from one cigar (average weight 5–6 g.), and pipe tobacco smoke from 6 g. tobacco. The time taken for the smoking was suitably adjusted.

The U-tube and flask were washed out with *N*/10 hydrochloric acid to dissolve the deposit, and the extract was filtered. It was then made alkaline with KOH and extracted with ether. The ether extract was poured into an evaporating basin and left overnight. The solid deposit was dissolved in 1 per cent tartaric acid (a few drops of alcohol assisted the process). It was then filtered and estimated. The estimations were made by comparing the rise of blood pressure of the spinal cat obtained after injecting doses of the extract with the rise obtained after injecting known doses of nicotine acid tartrate. The dose required and the time interval between injections varied in different cats. A dose of nicotine acid tartrate between 0.5–1.0 mg. was usually suitable, and a time interval of 5–7 min. was usually sufficient to avoid a decrease in sensitivity.

This was the method used by H. W. L., and the method of C. B. W. P. was essentially the same. There were two modifications in the later work. Firstly, a set number of aspirations, namely 12, were made in ten minutes for each cigarette, and, secondly, the extract after being made alkaline was extracted five times with ether. It is possible that a more thorough extraction with ether accounts for the slightly higher figures obtained by C. B. W. P.

Results

1. *Obtained by H. W. L. in 1935:*

A. Cigarettes

Each cigarette weighed approx. 1 g.

Brand	Number of cigarettes used to prepare extract	Amount of nicotine (base) per cigarette
Players	5	0.66 mg.
Wills Gold Flake..	10	0.75 mg.
,, ,, ,, ..	10	0.7 mg.

B. Cigarette tobacco

Wills Gold Flake, 0.6 mg. per g. tobacco.

C. Cigars

2 Marcella cigars, 3.8 mg. per cigar (each approx. 5 g.).

2. *Obtained by C. B. W. P. in 1948:*

A. Cigarettes

Brand	Number of cigarettes used to prepare extract	Amount of nicotine (base) mg. per cigarette
Players	5	0.83
,, ...	5	0.8
,, ...	5	0.94
,, ...	5	1.00
,, ...	5	0.8
State Express 555	5	0.72
,, ,,	5	1.00
,, ,,	5	0.98
,, ,,	5	1.20
	Mean	0.92

B. Cigars

(*a*) 3.6 mg. per cigar (weight about 5 g.).
(*b*) 6.26 mg. per cigar.
(*c*) 5.0 mg. per cigar.
(*d*) 7.9 mg. per cigar.
(*e*) 5.0 mg. per cigar.

C. Pipe tobacco

Four extracts of 6 g. each pipe tobacco were estimated. The results were:

(*a*) 2 mg. nicotine per g. tobacco.
(*b*) 2.75 mg. ,, ,, ,, ,,
(*c*) 3.0 mg. ,, ,, ,, ,,
(*d*) 3.0 mg. ,, ,, ,, ,,
Mean = 2.69 mg. ,, ,, ,, ,,

Discussion and Summary

The results show that in two independent investigations made at a time interval of 13 years the amount of nicotine found to be deposited in the smoke from well-known brands of cigarettes was of the same order. The war period has certainly not led to any diminution in the nicotine content. If all the results are considered, a figure between 0.6 mg. and 1.0 mg. per cigarette or per gramme tobacco is obtained. The cigars yielded between 3.6 mg. and 7.9 mg. per cigar, or 0.7 mg.–1.6 mg. per g. tobacco. The pipe tobacco yielded between 2 and 3 mg. nicotine per g. tobacco. This figure for pipe tobacco is higher than the figures for either cigarettes or cigars.

These figures give the amount of nicotine entering the mouth of the smoker. The proportion of nicotine absorbed into the circulation depends on the extent to which the smoker inhales, for nicotine in the smoke entering the alveoli is mostly absorbed. Absorption must also occur to some extent in the mucous membranes of the mouth, nose, and throat, for smoking without conscious inhaling raises the pulse rate and the blood pressure. Burn, Truelove, and Burn (1945) describe an experiment in which the same individual drank 1 litre of water on two occasions. The diuresis was then recorded by collecting urine at 15 min. intervals. The anti-diuretic effect of smoking 1 cigarette was of shorter duration than that produced by the intravenous injection of 0.5 mg. nicotine (base). Thus, although the smoker inhaled, less than 0.5 mg. nicotine (base) was absorbed into the circulation from one cigarette.

It can, therefore, be concluded that, while the amount of nicotine from one cigarette entering the mouth is about 0.9 mg., the amount absorbed is about 0.4 mg. or less.

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